



PHD

Characterisation of the nicotinic acetylcholine receptor from *Manduca sexta*

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Award date:
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Characterisation of the nicotinic acetylcholine receptor from *Manduca sexta*

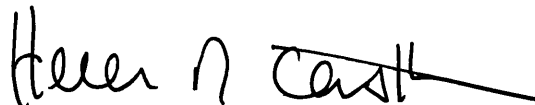
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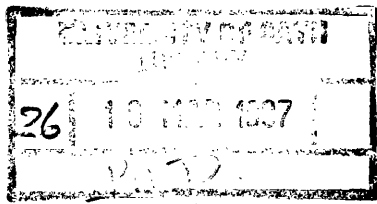
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ABBREVIATIONS

Standard abbreviations used in text are as defined in "Instructions to Authors" of the Biochemical Journal 1996.

| | |
|------------------|---|
| 5HT | 5-hydroxytryptamine |
| α BgtBP | α -Bungarotoxin Binding Protein |
| α -Btx | α -Bungarotoxin |
| ACh | Acetylcholine |
| AChE | Acetylcholinesterase |
| AChR | Acetylcholine Receptor |
| α L1/ARL2 | α -like subunit 1 of the nAChR from <i>Schistocerca gregaria</i> |
| ALS | α -like subunit 1 of the nAChR from <i>Drosophila melanogaster</i> |
| AnTx | anatoxin-a |
| ARD | β -like subunit 1 of the nAChR from <i>Drosophila melanogaster</i> |
| ARL1 | β -like subunit 1 of the nAChR from <i>Schistocerca gregaria</i> |
| B _{max} | maximal binding |
| ChAT | Choline acetyltransferase |
| CIAP | calf intestinal alkaline phosphatase |
| D α 2/SAD | α -like subunit 2 of the nAChR from <i>Drosophila melanogaster</i> |
| D α 3 | α -like subunit 3 of the nAChR from <i>Drosophila melanogaster</i> |
| DDF | p-(N,N-dimethylamino)benzenediazonium fluoroborate |
| DEPC | diethylpyrocarbonate |
| DIG | digoxigenin |
| DTT | dithiothreitol |
| EPC | endplate current |
| EPSP | excitatory postsynaptic potential |
| GABA | γ -aminobutyric acid |
| GC | gas chromatography |
| gcg | Genetics Computer Group (at the University of Wisconsin) |
| HAnTx | homoanatoxin |
| IPAnTx | isopropylanatoxin |
| IPN | interpeduncular nucleus |
| K _d | dissociation constant |

| | |
|----------------|--|
| K _i | inhibition constant |
| LGIC | ligand gated ion channel |
| Lumigen PPD | [4-methoxy-4-(3-phosphate-phenyl)-spiro(1,2-dioxetane-3,2'adamantane) disodium salt] |
| MAb | monoclonal antibody |
| mAChR | muscarinic Acetylcholine Receptor |
| MARA1 | <u>M</u> anduca <u>s</u> exta <u>A</u> cetylcholine <u>R</u> eceptor <u>A</u> lpha-like subunit <u>1</u> |
| MARB1 | <u>M</u> anduca <u>s</u> exta <u>A</u> cetylcholine <u>R</u> eceptor <u>B</u> eta-like subunit <u>1</u> |
| MBTA | 4-(N-maleimido)-benzyltrimethylammonium |
| MLA | methyllycaconitine |
| nAChR | nicotinic Acetylcholine Receptor |
| NBT | nitroblue tetrazolium |
| NCB | non-competitive blocker |
| nH | Hill number |
| NMN | N'methylnicotinamide |
| PAnTx | propylanatoxin |
| PEG | polyethylene glycol |
| PEI | polyethyleneimine |
| pI | isoelectric point |
| PTFE | polytetrafluoroethane |
| QNB | quinuclidinyl benzilate |
| RACE | rapid amplification of cDNA ends |
| SBD | β-like subunit 2 of the nAChR from <i>Drosophila melanogaster</i> |
| SM | storage medium for bacteriophage λ (see Appendix 3) |
| SSC | sodium chloride, sodium citrate solution (see Appendix 3) |
| TAE | Tris Acetate EDTA solution (see Appendix 3) |
| Taq | <i>Thermus aquaticus</i> |
| TE | Tris EDTA solution (see Appendix 3) |
| TM | transmembrane domain |
| TMA | tetramethylammonium |
| X-phosphate | 5-bromo-4-chloro-3-indolyl phosphate |

SUMMARY

Manduca sexta is a nicotine insensitive insect, the larval form of which feeds on tobacco. It was postulated that the nicotine insensitivity may be due, in part, to a modified nicotinic acetylcholine receptor (nAChR) whose α subunit does not possess the amino acid residues necessary to bind nicotine. The aim of this study was to test this hypothesis.

A partial length genomic clone and a partial length cDNA clone were isolated using inverse PCR on circularised genomic DNA and cDNA respectively. Together with an overlapping partial length cDNA clone previously isolated (Clarke, 1991), they encode the complete coding sequence of an α -like subunit of nAChR which does not show any obvious candidate amino acids substituting for those residues known to take part in nicotine binding in vertebrates. This α -like subunit is named MARA1 for *Manduca* Acetylcholine Receptor Alpha-like subunit 1. Further searches have not isolated other α -like subunits, suggesting that only one α -like subunit may exist in *Manduca*. Proof that MARA1 was nicotine sensitive would require functional analysis. Therefore, two-electrode voltage clamp recording on *Xenopus* oocytes expressing the mouse muscle nAChR was carried out with the intention of using this technique for a functional assessment of MARA1. However, this was not feasible because of the difficulty in obtaining a full-length clone either *de novo* or by ligation of the overlapping clones, possibly due to complex RNA secondary structure.

$[^{125}\text{I}]\alpha$ -Bungarotoxin ($[^{125}\text{I}]\alpha$ -btx) binding was performed to investigate the presence of nicotine binding sites. Specific $[^{125}\text{I}]\alpha$ -btx binding was demonstrated in both *Manduca* larval and adult membranes, consistent with an α -btx sensitive nAChR. Inhibition of $[^{125}\text{I}]\alpha$ -btx binding by cholinergic ligands suggested that the same $[^{125}\text{I}]\alpha$ -btx binding site was present in larvae and adults. Furthermore, this $[^{125}\text{I}]\alpha$ -btx binding site bound nicotine (K_i values of $1.6\mu\text{M}$ and $2\mu\text{M}$ for larvae and adults respectively) with similar affinity to nicotine-sensitive insects, indicating that a modified nAChR is unlikely to be the definitive mechanism in *Manduca*'s nicotine insensitivity. This corroborates the sequence data from MARA1. $[^3\text{H}](\pm)$ Epibatidine binding on *Manduca* larval and adult membranes did not reveal the presence of an α -btx insensitive binding site, consistent with the isolation of only one α -like subunit from *Manduca*.

Sequence and binding data predict that there is not a modified nAChR in *Manduca* and therefore other mechanisms of protection against nicotine toxicity in *Manduca* must exist.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr Susan Wonnacott and Dr Adrian Wolstenholme for their help and advice throughout this project. I would also like to thank Professor Stuart Reynolds for his *Manduca* dissection demonstrations, Dr Susan Wheeler for teaching me the basics of two electrode voltage clamp recording from *Xenopus* oocytes and Ewan Basterfield for his care of the *Xenopus* colony.

Many, many thanks go to Robert Lind for his excellent collaboration in the *Manduca* binding studies and for the photographs of *Manduca*. Thanks also to Dr Paul Whiteaker for explaining the complexities of SigmaPlot.

I am eternally grateful to all my family for their consistent support and encouragement and most of all to Ross for listening.

This project was supported by an EC Biotechnology grant.

CONTENTS

| | |
|---|--------------|
| Title and copyright declaration | i |
| Abbreviations | ii |
| Summary | iv |
| Acknowledgements | v |
| Contents | vi |
| Figures | xiii |
| Tables | xvi |
| Chapter 1. Introduction | 1 |
| 1.1 <i>Manduca sexta</i> : a nicotine resistant insect | 2 |
| 1.1.1 Nicotine as an insecticide | 2 |
| 1.1.2 <i>Manduca sexta</i> | 3 |
| 1.1.3 Protective mechanisms against nicotine in <i>Manduca</i> | 6 |
| Metabolism and excretion in <i>Manduca</i> | 6 |
| Protective adaptations of the <i>Manduca</i> central nervous system | 8 |
| Metabolism in the central nervous system | 9 |
| Barriers to nicotine penetration | 10 |
| Selective transport | 11 |
| Receptor modification | 11 |
| Summary | 13 |
| 1.2 Vertebrate nicotinic acetylcholine receptors (nAChRs) | 14 |
| 1.2.1 The ligand gated ion channel family | 14 |
| 1.2.2 Muscle-type nAChRs | 14 |
| 1.2.3 Conserved structural features of the nAChR | 15 |
| 1.2.4 Neuronal-type nAChRs | 16 |
| Cloning of genes and cDNAs encoding neuronal nAChRs | 16 |
| 1.2.5 Confirmation of function in neuronal nAChRs | 17 |
| Purification of neuronal nAChRs | 17 |
| Functional expression of neuronal nAChR genes | 19 |
| Functional diversity of expressed nAChRs | 21 |
| Correlating subunit composition to native receptors | 22 |
| 1.2.6 Presynaptic nAChR | 22 |
| 1.2.7 Agonist and antagonist binding sites | 23 |
| Localisation of binding sites | 23 |
| Structural features of the nAChR | 24 |
| Structure of the ACh binding site | 24 |

| | |
|--|---------------|
| The cysteine loop | 25 |
| Functional appearance of the ACh binding site | 27 |
| Contribution of non- α subunits to the ACh binding site | 28 |
| Non-competitive agonists | 29 |
| The α -bungarotoxin binding site | 30 |
| The ion channel | 32 |
| Phosphorylation of the nAChR | 33 |
| 1.3 Insect nAChRs | 35 |
| 1.3.1 Binding sites for nicotinic cholinergic ligands in the central nervous system | 35 |
| Pharmacological characterisation | 35 |
| Biochemical characterisation and reconstitution into lipid bilayers | 36 |
| Autoradiographic localisation | 37 |
| Electrophysiological characterisation | 38 |
| 1.3.2 Cloning of genes and cDNAs that encode insect nAChRs | 39 |
| 1.3.3 Functional analysis of cloned genes and cDNAs | 46 |
| Immunoprecipitation of [125 I] α -bungarotoxin binding sites | 46 |
| Expression in <i>Xenopus</i> oocytes | 47 |
| Localisation by immunohistochemical studies | 49 |
| Temporal and spatial localisation | 50 |
| 1.4 Summary | 51 |
| Chapter 2. Molecular characterisation of an α-like subunit of the nAChR from <i>Manduca sexta</i> | 52 |
| 2.1 Introduction | 53 |
| 2.2 Materials | 55 |
| 2.3 Methods | 57 |
| 2.3.1 Techniques used in nucleic acid preparation and synthesis | 57 |
| Calcium chloride competent cells | 57 |
| Transformation | 57 |
| α -Complementation | 57 |
| Storage of bacterial cultures | 57 |
| Plasmid DNA preparation | 57 |
| Mini scale | 57 |
| Midi scale | 58 |
| Genomic DNA preparation | 58 |
| Total RNA isolation | 59 |
| Poly [A+] RNA isolation | 59 |

| | |
|---|----|
| First strand cDNA synthesis | 60 |
| Second strand cDNA synthesis and circularisation | 60 |
| Single stranded cDNA preparation | 61 |
| 2.3.2 Techniques used in the purification of nucleic acids | 61 |
| Extraction of nucleic acids with phenol:chloroform | 61 |
| Concentration of nucleic acids by ethanol precipitation | 61 |
| Purification of DNA from agarose gels | 61 |
| Removal of short fragments of DNA | 61 |
| 2.3.3 Techniques used in the analysis of nucleic acids | 61 |
| Estimation of concentration of nucleic acid | 61 |
| Agarose gel electrophoresis | 62 |
| Denaturing gels | 62 |
| Sequencing | 62 |
| 2.3.4 Techniques used in the manipulation of nucleic acids | 63 |
| Restriction digests | 63 |
| Plasmid DNA digests | 63 |
| Genomic DNA digests | 63 |
| Ligations | 63 |
| Ligations using T4 DNA Ligase | 63 |
| Ligations using T4 RNA Ligase | 63 |
| Basic polymerase chain reactions (PCR) | 64 |
| Subcloning PCR products | 65 |
| 2.3.5 Techniques used in radioactive hybridisation | 65 |
| Southern blots | 65 |
| Radiolabelling of probes | 66 |
| Purification of radiolabelled probes | 66 |
| Hybridisation | 66 |
| Stripping the blot of radiolabelled probe | 66 |
| 2.3.6 Techniques used in non-radioactive hybridisation for screening of a cDNA library | 67 |
| Plating out | 67 |
| Plaque lifts | 67 |
| Probe preparation | 67 |
| Oligonucleotide 3' tailing | 67 |
| Random prime labelling | 68 |
| Hybridisation | 68 |
| Washing | 68 |
| Detection | 68 |

| | |
|--|-----|
| 2.3.7 Oligonucleotides | 69 |
| In house synthesis and deprotection of oligonucleotides | 69 |
| Radiolabelling of oligonucleotides | 69 |
| 2.4 Results | 70 |
| 2.4.1 Screening of the cDNA library | 70 |
| 2.4.1.1 Initial characterisation of the cDNA library | 70 |
| PCR on the <i>Manduca</i> library | 70 |
| Calculating the library titre | 70 |
| Determining the range of insert size | 70 |
| 2.4.1.2 Results of the library screen | 71 |
| Identification of positive plaques | 71 |
| Nested PCR on the <i>Manduca</i> cDNA library | 71 |
| 2.4.2 Inverse PCR on double stranded, circularised cDNA | 74 |
| 2.4.2.1 Analysis of preparative steps and inverse PCR products | 75 |
| 2.4.2.2 Sequencing analysis of inverse PCR product | 75 |
| 2.4.3 Ligation anchored PCR | 79 |
| 2.4.4 Inverse PCR on circularised, genomic DNA | 82 |
| 2.4.4.1 Analysis of the genomic DNA | 82 |
| 2.4.4.2 Inverse PCR on digested, circularised genomic DNA | 83 |
| 2.4.4.3 Sequencing analysis | 85 |
| 2.4.5 Attempts to obtain a full-length MARA1 | 87 |
| 2.4.5.1 Preliminary observations | 87 |
| Southern blot analysis | 88 |
| PCR analysis | 90 |
| 2.4.5.2 Searching for a full-length clone | 92 |
| An embryonic developmental profile | 92 |
| Searching for a <i>de novo</i> copy | 95 |
| Ligation of fragments a and c | 97 |
| Searching for a genomic clone | 99 |
| 2.4.6 Searching for other α -like subunits | 103 |
| 2.5 Discussion | 108 |
| 2.5.1 Method analysis | 108 |
| Searching for a full-length MARA1 clone | 110 |
| 2.5.2 Possible causes for the lack of a full-length MARA1 | 111 |
| 2.5.3 Analysis of the MARA1 sequence | 113 |
| Homology with other α -like subunits | 118 |
| Homomeric versus heteromeric receptors | 120 |
| Agonist and antagonist binding | 122 |

| | |
|---|------------|
| Chapter 3. Pharmacological characterisation of the nAChR from | |
| <i>Manduca sexta</i> | 127 |
| 3.1 Introduction | 128 |
| 3.1.1 The relationship between radiolabelled binding sites and cloned subunits | 128 |
| $[^3\text{H}]$ Nicotine | 128 |
| $[^{125}\text{I}]\alpha$ -Bungarotoxin | 129 |
| 3.1.2 AChRs in Insects | 130 |
| Nicotinic AChRs | 130 |
| Mixed AChRs | 130 |
| Muscarinic AChRs | 131 |
| <i>Manduca</i> AChRs | 131 |
| 3.1.3 Characteristics of specific binding | 132 |
| 3.2 Materials | 134 |
| 3.3 Methods | 135 |
| 3.3.1 Membrane preparation | 135 |
| Preparation of adult P2 membranes | 135 |
| Preparation of larval P2 membranes | 135 |
| 3.3.2 Protein determination | 136 |
| 3.3.3 $[^{125}\text{I}]\alpha$-Bungarotoxin GF/C filter binding assays | 136 |
| 3.3.3.1 Protein dependence of $[^{125}\text{I}]\alpha$ -bungarotoxin binding | 137 |
| 3.3.3.2 Time and temperature dependence of $[^{125}\text{I}]\alpha$ -bungarotoxin binding | 137 |
| 3.3.3.3 Saturation binding | 137 |
| 3.3.3.4 $[^{125}\text{I}]\alpha$ -Bungarotoxin competition binding assays | 137 |
| Pre-incubation of (-)nicotine | 138 |
| 3.3.4 $[^3\text{H}](\pm)$Epibatidine binding assays | 138 |
| 3.3.5 Data analysis | 138 |
| 3.4 Results | 140 |
| 3.4.1 Optimisation of $[^{125}\text{I}]\alpha$-bungarotoxin binding | 140 |
| Membrane preparations | 140 |
| $[^{125}\text{I}]\alpha$ -Bungarotoxin binding | 140 |
| Protein dependence of $[^{125}\text{I}]\alpha$ -bungarotoxin binding | 140 |
| Time and temperature dependence of $[^{125}\text{I}]\alpha$ -bungarotoxin binding | 142 |
| 3.4.2 Saturation binding of $[^{125}\text{I}]\alpha$-bungarotoxin binding | 142 |
| 3.4.3 $[^{125}\text{I}]\alpha$-Bungarotoxin competition binding | 144 |

| | |
|---|------------|
| Assessment of (-)-nicotine pre-incubation time on the inhibition of [¹²⁵ I]α-bungarotoxin binding | 144 |
| Assessment of inhibition by acetylcholinesterase inhibitors | 146 |
| Inhibition by nicotinic ligands | 147 |
| Inhibition by muscarinic ligands | 150 |
| 3.4.4 [³ H](±)Epibatidine competition binding | 151 |
| 3.5 Discussion | 153 |
| Saturability of α-btx binding sites | 153 |
| Metabolism of nicotine by the central nervous system in <i>Manduca</i> | 153 |
| Pharmacological specificity | 155 |
| Comparison with other insects | 156 |
| The nicotinoids - nicotine and anabasine | 156 |
| Neonicotinoids - imidacloprid | 159 |
| Acetylcholine and acetylcholinesterase inhibitors | 161 |
| Binding of antagonists | 163 |
| Is there a [³ H](±)epibatidine binding site in <i>Manduca</i> ? | 164 |
| Interpretation of Hill numbers | 165 |
| Muscarinic and mixed receptors in <i>Manduca</i> | 167 |
| Chapter 4. An overall perspective | 169 |
| 4.1 Relating sequence and binding data | 170 |
| 4.2 Future directions | 172 |
| 4.3 Why is <i>Manduca</i> resistant to nicotine? | 173 |
| Chapter 5. Two electrode voltage clamp recording from <i>Xenopus</i> oocytes | 177 |
| 5.1 Introduction | 178 |
| 5.1.1 Two electrode voltage clamp | 179 |
| 5.1.2 The <i>Xenopus</i> oocyte expression system | 179 |
| 5.1.3 The muscle nAChR | 180 |
| 5.1.4 Anatoxin and methyllycaconitine | 181 |
| Anatoxin-a | 181 |
| Anatoxin-a analogues | 181 |
| Methyllycaconitine | 182 |
| 5.1.5 Aims | 183 |
| 5.2 Materials | 184 |
| 5.3 Methods | 185 |
| 5.3.1 <i>In vitro</i> transcription | 185 |

| | |
|--|---------|
| 5.3.2 <i>Xenopus</i> and <i>Xenopus</i> oocytes | 186 |
| <i>Xenopus</i> maintenance | 186 |
| Preparation of oocytes | 186 |
| 5.3.3 Two electrode voltage clamp | 187 |
| 5.4 Results | 188 |
| 5.5 Discussion | 194 |
| Appendix 1. <i>Torpedo californica</i> α subunit sequence | 199 |
| Appendix 2. Primers | 200 |
| Appendix 3. Solutions | 202 |
| References | 203 |

FIGURES

| | | |
|------|---|----|
| 1.1 | Stages of the <i>Manduca sexta</i> lifecycle | 5 |
| 1.2 | Metabolic products formed from the breakdown of nicotine in the midgut of <i>Manduca</i> larvae | 7 |
| 1.3 | Structure of the nAChR | 26 |
| 1.4 | Proposed subunit arrangement around the ion channel for nAChRs expressed in <i>Xenopus</i> oocytes | 28 |
| 1.5 | Sequence alignment of α 180-200 from α -btx sensitive and α -btx insensitive subunits indicating residues postulated to form interactions with α -btx | 32 |
| 1.6 | Alignment of insect α -like and β -like subunits of the nAChR | 42 |
| 1.7 | Comparison of intron positions for genes encoding <i>Drosophila</i> α -like and β -like subunits and vertebrate neuronal and muscle subunits | 45 |
| 2.1 | PCR on the <i>Manduca</i> cDNA library to confirm the presence of an α -like subunit in the library | 73 |
| 2.2 | PCR on randomly picked plaques from the library screen to determine range of insert size | 73 |
| 2.3 | Nested PCR on <i>Manduca</i> cDNA library (schematic) | 72 |
| 2.4 | Nested PCR on the <i>Manduca</i> cDNA library | 73 |
| 2.5 | Inverse PCR on double stranded, circularised cDNA (schematic) | 74 |
| 2.6 | Analysis of first strand cDNA synthesised from RNA isolated from <i>Manduca</i> eggs | 76 |
| 2.7 | Inverse PCR on double stranded, circularised cDNA | 76 |
| 2.8 | Nucleotide and amino acid sequence of fragment b | 77 |
| 2.9 | Alignment of part of fragment b with part of the sequence encoding α L1 (ARL2) from <i>Schistocerca</i> and D α 2/SAD from <i>Drosophila</i> | 78 |
| 2.10 | Ligation anchored PCR on first strand PCR (schematic) | 79 |
| 2.11 | Ligation anchored PCR | 81 |
| 2.12 | Inverse PCR on genomic DNA (schematic) | 82 |
| 2.13 | Genomic DNA digests | 84 |
| 2.14 | Southern blot of <i>Manduca</i> genomic DNA | 84 |
| 2.15 | PCR on <i>Hinc</i> II digested and circularised genomic DNA from <i>Manduca</i> | 84 |
| 2.16 | Nucleotide and amino acid sequence of fragment a | 85 |
| 2.17 | Alignment of part of fragment a with part of the sequences encoding ALS from <i>Drosophila</i> and α L1 (ARL2) from <i>Schistocerca</i> | 86 |
| 2.18 | Autoradiograph from the hybridisation of Southern blots of digested | |

| | |
|--|-----|
| genomic DNA with each fragment encoding the α -like subunit of the nAChR from <i>Manduca</i> | 89 |
| 2.19 PCR to splice together fragments a and c | 91 |
| 2.20 Nucleotide and amino acid sequence of fragment d | 90 |
| 2.21 Nested PCR on first strand cDNA | 94 |
| 2.22 Autoradiograph of Southern blot of nested PCR on first strand cDNA | 94 |
| 2.23 PCR on first strand cDNA using primers specific to the extreme ends of sequence encoding the MARA1 subunit | 96 |
| 2.24 Splicing by the overlap extension method (schematic) | 97 |
| 2.25 Splicing by the megaprimer method (schematic) | 98 |
| 2.26 Splicing by engineered restriction sites (schematic) | 99 |
| 2.27 PCR on genomic DNA using primers specific to extreme ends of the sequence encoding MARA1 | 101 |
| 2.28 Partial nucleotide sequence of the genomic PCR product | 100 |
| 2.29 Alignment of part of genomic DNA PCR product with non-nAChR sequences | 102 |
| 2.30 Amplification of further putative α -like subunits of the nAChR by PCR with degenerate primers on cDNA | 104 |
| 2.31 Autoradiograph of Southern blot of PCR with degenerate primers on cDNA | 104 |
| 2.32 Nucleotide sequence of PCR product obtained with degenerate primers on first strand cDNA | 105 |
| 2.33 Alignment of PCR product obtained with degenerate primers on cDNA with non-nAChR sequences | 105 |
| 2.34 Nucleotide and amino acid sequence of MARA1 | 107 |
| 2.35 Optimal RNA secondary structure prediction for the extracellular region of MARA1 | 112 |
| 2.36 Alignment of insect α -like sequences | 117 |
| 2.37 Phylogenetic tree of insect α -like subunits | 119 |
| 2.38 Residues in the extracellular region of MARA1, potentially involved in ligand binding | 124 |
| 2.39 Alignment of potential α -btx binding site | 125 |
| 3.1 Protein dependence of [125 I] α -btx binding to P2 membranes | 141 |
| 3.2 Time and temperature dependence of [125 I] α -btx binding | 143 |
| 3.3 Saturable [125 I] α -btx binding to P2 membranes at the [125 I] α -btx binding site in adults and larvae | 143 |
| 3.4 Competition binding assay of α -btx at the [125 I] α -btx binding site in adults and larvae | 145 |

| | | |
|-----|---|-----|
| 3.5 | Effect of pre-incubation on the inhibition of specific [125 I] α -btx binding by (-)nicotine | 145 |
| 3.6 | Inhibition of specific [125 I] α -btx binding by acetylcholinesterase inhibitors | 146 |
| 3.7 | Competition binding assays of cholinergic ligands at the [125 I] α -btx binding site in adults and larvae | 148 |
| 3.8 | Inhibition of specific [125 I] α -btx binding by muscarinic ligands for adults and for larvae | 151 |
| 3.9 | Inhibition of [3 H](\pm)epibatidine binding by excess (-)nicotine and α -btx in adult and larval membranes | 152 |
| 4.1 | The route taken by nicotine to nAChRs in the CNS of insects when used as an insecticide | 175 |
| 5.1 | Schematic of a <i>Xenopus</i> oocyte | 179 |
| 5.2 | <i>In vitro</i> transcription of mouse muscle nAChR subunits | 190 |
| 5.3 | Activation of mouse muscle nAChRs expressed in <i>Xenopus</i> oocytes by ACh, AnTx and AnTx analogues | 191 |
| 5.4 | Experimental analysis of activation of mouse muscle nAChR expressed in <i>Xenopus</i> oocytes by ACh, AnTx and AnTx analogues | 192 |
| 5.5 | Inhibition of mouse muscle nAChRs expressed in <i>Xenopus</i> oocytes by MLA | 193 |

TABLES

| | | |
|-----|---|-----|
| 1.1 | ED ₅₀ values for cholinergic agonists on the PPR motoneuron from <i>Manduca</i> and the Df motoneuron from <i>Periplaneta</i> | 9 |
| 1.2 | Potential protective mechanisms against nicotine in <i>Manduca</i> | 13 |
| 1.3 | Amino acid identities between cDNA encoding muscle and neuronal subunits of chick and rat | 17 |
| 1.4 | Published insect cDNA sequences encoding nAChR subunits | 41 |
| 1.5 | Amino acid identities between cDNA encoding insect α -like and β -like subunits | 44 |
| 1.6 | Expression of insect subunits in <i>Xenopus</i> oocytes | 49 |
| 3.1 | Parameters for saturation binding from adult and larval P2 membranes | 142 |
| 3.2 | Effect of nicotine pre-incubation time on the displacement of [¹²⁵ I] α -btx binding as measured by K _i values | 144 |
| 3.3 | Inhibition of [¹²⁵ I] α -btx binding by acetylcholinesterase inhibitors | 146 |
| 3.4 | Parameters for the inhibition of specific [¹²⁵ I] α -btx binding by cholinergic ligands | 147 |
| 3.5 | Statistical significance of K _i values for cholinergic ligands between adults and larvae | 149 |
| 3.6 | Hill numbers derived from inhibition of specific [¹²⁵ I] α -btx binding by cholinergic ligands in adults and larvae | 150 |
| 3.7 | Comparison of B _{max} and K _d for <i>Manduca</i> , <i>Apis</i> and <i>Schistocerca</i> | 153 |
| 3.8 | Comparison of <i>Manduca</i> K _i values with those from <i>Drosophila</i> , <i>Musca</i> , <i>Apis</i> , <i>Periplaneta</i> , <i>Schistocerca</i> and <i>Locusta</i> | 157 |
| 5.1 | EC ₅₀ values for mouse muscle and chick α 7 nAChRs | 195 |

CHAPTER 1

Introduction

1.1 *Manduca sexta*: a nicotine resistant insect

1.1.1 Nicotine as an insecticide

The insecticidal properties of the tobacco plant *Nicotiana tabacum* have been known for centuries. However, not until 1828, when nicotine, the active alkaloid was isolated (Jackson, 1941), did commercial preparations containing standard nicotine contents become available. At its peak in 1944, the equivalent of 1.2 million pounds of nicotine was used in agriculture in the USA (Metcalf, 1948). Today, nicotine has limited use as an insecticide compared with the inorganic insecticides such as arsenicals and fluorides and the synthetic organic insecticides such as chlorinated hydrocarbons and organophosphorus and carbamate acetylcholinesterase inhibitors. This is due to its high mammalian toxicity, its narrow insecticidal spectrum (only being effective against soft-bodied insects with piercing mouthparts such as aphids) and its short life span under field conditions. Nicotine does offer some advantage over these other insecticides by acting on a different target in the nervous system of insects, thus insect strains which develop resistance to, say, acetylcholinesterase inhibitors are unlikely to develop cross-resistance to nicotine (reviewed in M. Eldefrawi, 1985).

Acetylcholine (ACh) is the major excitatory neurotransmitter in the insect central nervous system (CNS), where it acts on acetylcholine receptors (AChRs) (reviewed in Colhoun, 1963 and Sattelle, 1980). The insecticidal activity of nicotine is due to its interaction with AChRs and in particular with the nicotinic AChR (nAChR), as opposed to the muscarinic AChR (mAChR), G-protein coupled receptors that are discussed in section 1.2.1. In the vertebrate peripheral nervous system (PNS) and the insect CNS, binding of ACh to nAChRs causes a transient conformational change in the receptor which creates a temporary channel through the protein and provides an aqueous environment for the passage of the cations Na^+ and K^+ across the membrane. The excitable cell membrane is consequently depolarised and an action potential elicited if a threshold level is reached, upon which activation is terminated and the receptor returned to its resting state. Under normal conditions, the ACh is rapidly degraded by membrane bound and soluble acetylcholinesterase (AChE), thus preventing re-activation of the receptor. Similarly, nicotine binds to nAChRs, activating ion channel opening and subsequently causing an influx of (predominantly) sodium ions. This depolarisation initiates multiple firing of action potentials and excitation. Eventually, the prolonged activation of the ion channels results in a depolarisation of the initiating membrane that is sufficiently large to inactivate a major proportion of the ion channels responsible for the action potential. Consequently, too few ion channels remain in the resting and thus activatable state to

generate an action potential and the conduction of nerve impulses fails. This is known as a "depolarising block" (Lund, 1985) and is the fundamental mechanism behind the toxicity of many cholinergic drugs. The toxic effects of nicotine observed in animals are generally due to a "depolarising blockade" of the nAChRs in the PNS, especially those of the respiratory muscles which leads to respiratory paralysis. In insects however, nicotine exerts its toxic effects upon nAChRs in the CNS. This mechanism is not necessarily applicable to the mammalian CNS due to the presence of presynaptic nAChR (see section 1.2.6).

1.1.2 *Manduca sexta*

Tobacco presumably contains nicotine as a defence against predators (i.e. nicotine acts as an allelochemical). This results in repelling the majority of insects but conversely attracts those which are adapted to nicotine ingestion, one such example being the larvae of the tobacco hornworm, *Manduca sexta* (Lepidoptera; Sphingidae), which feed by leaf chewing and can tolerate diets containing 1-1.5% (wet weight) nicotine (Parr and Thurston, 1972). Incredibly, these larvae are also able to tolerate alkaloid ingestion from other solanaceous plants, including tomato, deadly nightshade and jimson weed. Thus for those plants that are economically important, *Manduca* can be a considerable pest. Accordingly, larvae of *Manduca* have been the target for insecticides on agricultural crops. As with other lepidopterans the adult moth feeds on nutritious liquids such as nectar from live and decaying plants and thus is a lesser pest than the larvae. The stages of the *Manduca* lifecycle are shown in Figure 1.1.

Initially, it could be postulated that a lack of nAChRs causes the nicotine resistance seen in *Manduca*. However, there is substantial evidence to suggest that ACh acts as an excitatory neurotransmitter at synapses in the *Manduca* CNS, in particular at those sensory neurons in the antennae which make synapses in the antennal lobes of the developing adult (pupae) brain (see Hildebrand *et al.*, 1980 for detailed explanation of brain structure). It has been shown that antennae incubated with [¹⁴C]choline can synthesize and store [¹⁴C]ACh whereas several other neurotransmitter candidates do not accumulate when supplied with the appropriate radiolabelled precursors. Also, antennae and antennal nerves contain endogenous ACh, while extracts of antennae contain choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), the biosynthetic and degradative enzymes of ACh respectively. Furthermore, levels of ACh, ChAT and AChE all rise during development and reach a plateau when neurons become morphologically mature (Sanes and Hildebrand, 1976). Similar experiments following quantification of ACh, ChAT and AChE from postembryonic (larvae) to adult development have shown

that variations in activity (particularly of ChAT) correlate with known developmental endocrine events and hence indicate cholinergic neurochemical differentiation (Prescott *et al.*, 1977).

The presence of nAChRs has been suggested by an [125 I] α -bungarotoxin ([125 I] α -btx) binding activity (a snake venom which blocks nAChRs at the vertebrate neuromuscular junction; see section 1.2.2) which rises gradually during antennal lobe development, is specific to nervous tissue and can be blocked by cholinergic agents such as carbamylcholine, d-tubocurarine and nicotine. Moreover, this activity is not lost in lobes deafferented by amputation of developing antennae (Sanes *et al.*, 1977). The [125 I] α -btx binding sites have been located in condensed regions of the neuropile of antennal lobes, that are known to be rich in synapses (Hildebrand *et al.*, 1979).

Therefore, it appears that the presence and storage of ACh, the presence of ChAT and its ability to synthesise ACh from its precursors and the presence of an inactivating enzyme, AChE, together with a putative nicotine binding site suggests that ACh exerts a functional role in the CNS of *Manduca*. Hence it can be assumed that nAChRs, or at least nicotinic-like AChRs exist in *Manduca*. Consequently, an absence of the nAChR cannot explain the nicotine insensitivity of *Manduca* larvae (and possibly adult) and other mechanisms that protect against the toxic effects of nicotine must be considered.



Figure 1.1. Stages of the *Manduca sexta* lifecycle. From top to bottom, eggs and 1st instar larva (bar = 2mm), 5th instar larva, pupa and adult (bars = 1cm).

1.1.3 Protective mechanisms against nicotine in *Manduca*

Metabolism and excretion in *Manduca*

Early studies by Self *et al.* (1964a) showed that *Manduca* larvae could ingest at least 20mg of nicotine in 24 hours without showing signs of toxicity. This was attributed to the rapid excretion of unmetabolised nicotine as larvae were able to eliminate most of the ingested nicotine within 2 hours and no metabolites of nicotine, as determined by GC and paper chromatography, were found. A similar mechanism was found for two other lepidopterous, tobacco feeding insects, the tobacco budworm, *Heliothis virescens* and the cabbage looper, *Trichoplusia ni* (Self *et al.*, 1964b). In contrast, the tobacco wireworm, *Conoderus vespertinus* (a coleopteran) and nymphs of the differential grasshopper, *Melanoplus differentialis* (an orthopteran), were able to rapidly excrete nicotine metabolites, principally in the form of cotinine. This theory of tolerance by rapid excretion was reinforced by the finding that larval *Manduca* Malpighian tubules could rapidly remove nicotine by an active transport system that eliminated nicotine at a rate of approximately 3nmol/min, whereas the adult *Manduca* Malpighian tubules could only excrete nicotine by passive transport at a rate of approximately 4pmol/min (Maddrell and Gardiner, 1976). It was also established that larvae of the cabbage white butterfly, *Pieris brassicae* (another lepidoteran), could actively transport nicotine at high rates. However, as *Pieris brassicae* larvae were readily killed by dietary nicotine (David and Gardiner, 1953), high excretion rates alone could not account for *Manduca* larval adaptation to nicotine.

It has been shown that rapid metabolism followed by excretion are the major mechanisms for tolerance to chemicals such as xanthotoxin and α -terthienyl in some chemically resistant lepidopterans (Ivie *et al.*, 1983; Iyengar *et al.*, 1987). It is not so surprising, therefore, that the more modern techniques of mass spectrometry and NMR have revealed that the nicotine ingested by *Manduca* larvae is metabolised, predominantly to cotinine-N-oxide and partially to nicotine-1-N-oxide and cotinine (Snyder *et al.*, 1994). Metabolism occurs via N-oxidation to nicotine-1-N-oxide; C5' oxidation to cotinine and cotinine-N-oxide is probably formed from the nicotine-1-N-oxide, as shown in Figure 1.2. Both cotinine-N-oxide and nicotine-1-N-oxide are more polar than nicotine and this aids their excretion. Some alkaloid sequestration occurred, as the level of alkaloids still increased in the haemolymph and larvae 'carcass' after they had stabilised in the digestive tract. It was suggested that the rapid metabolism and excretion of nicotine by *Manduca* larvae could serve as a protective mechanism for the pupal and adult stages, as internal alkaloid levels could be eliminated quickly as feeding ceased in preparation for pupation.

As an aside, the authors attributed the lack of metabolism seen in *Manduca* by Self *et al.* (1964a) to the analytical techniques used. It was suggested that thermal deoxygenation of cotinine-N-oxide and nicotine-1-N-oxide would cause these compounds to behave like cotinine and nicotine respectively on a GC column. Furthermore, the paper chromatography would not have resolved cotinine-N-oxide, the major metabolite.

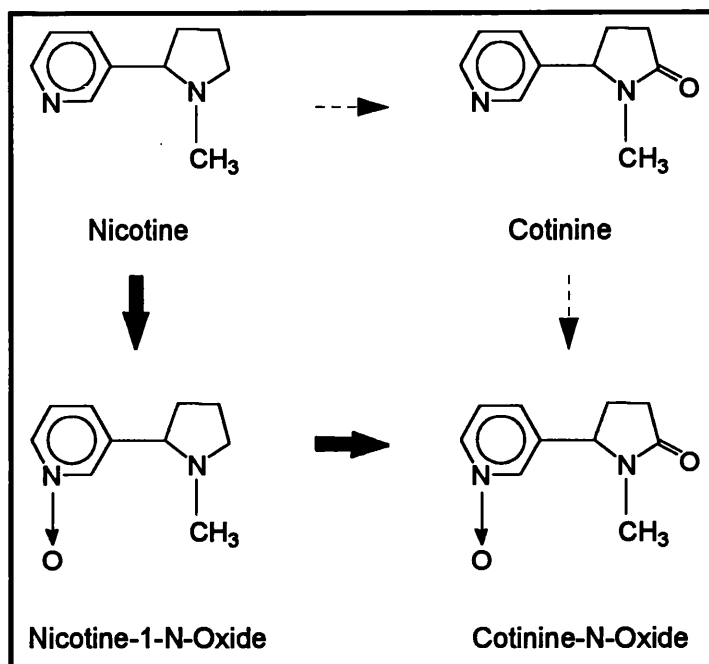


Figure 1.2. Metabolic products formed from the breakdown of nicotine in the midgut of *Manduca* larvae. Thick arrows represent major routes, dashed arrows represent minor routes. Redrawn from Snyder *et al.* (1994).

The changing pattern of metabolites in different regions of the larvae suggested that the dietary nicotine is able to induce its own metabolism, which was reversed on removal from a nicotine diet. A further study indicated that this induction of nicotine metabolism involved midgut microsomal cytochrome P-450 mono-oxygenases (Snyder *et al.*, 1993). It was shown that at a low dietary nicotine concentration (0.1%) three of 12 midgut microsomal P-450 enzyme activities were induced whereas at higher concentrations of nicotine, similar to those found in tobacco (0.75%), nine of 12 enzyme activities were induced, suggesting that a subset of P-450 genes was differentially expressed at low doses. Moreover, the induction of cytochrome P-450 appeared to have a protective effect, as those larvae previously fed nicotine displayed significantly faster nicotine metabolism. Thus, it appears that in *Manduca* larvae the potential toxicant nicotine, from the host plant tobacco, is able to induce its own metabolism by cytochrome P-450 enzymes. Interestingly, this also appears to be an adaptation to nicotine ingestion used by *Heliothis* (Rose *et al.*, 1991) and by the southern armyworm, *Spodoptera eridania*

(Brattsten *et al.*, 1977). Hence, this inducible system provides a means of defence against chemical stress.

Protective adaptations of the Manduca central nervous system

Although metabolism and excretion appear to be means of achieving chronic tolerance to nicotine ingestion in *Manduca*, electrophysiological recordings on *Manduca* nerve cord have indicated that local protective mechanisms must exist within the CNS. Global recordings on intact nerve cords from larvae have shown that the 10mM concentration of nicotine necessary to evoke a response is two orders of magnitude higher than that needed for the cockroach, *Periplaneta americana*, indicating that the *Manduca* CNS is approximately 100-fold less sensitive to nicotine than a nicotine sensitive insect, *Periplaneta* (Morris, 1984). In contrast, the cholinergic agonists ACh, muscarine, lobeline and tetramethylammonium (TMA) evoked responses at lower concentrations than nicotine, suggesting that the CNS of *Manduca* was not intrinsically insensitive to cholinergic agents. Desheathing of the nerve cord, resulting in the removal of the perineurium, increased the sensitivity of the *Manduca* CNS to nicotine but did not make the tissue as responsive as the *Periplaneta* CNS.

More detailed studies using intracellular recording methods on an isolated, desheathed motoneuron (PPR) in *Manduca* larvae have reinforced this earlier finding (Trimmer and Weeks, 1989). The synapse studied is part of the pathway mediating reflexive withdrawal of the abdominal prolegs. In this pathway, the reflex is generated by tactile stimulation of mechanosensory hairs (the planta hairs) located on the top of each proleg. The resultant withdrawal is initiated by contraction of the principal planta retractor muscle (PPRM) that is innervated by the single motoneuron, PPR. It was demonstrated that electrical stimulation of PPR elicited a monosynaptic excitatory postsynaptic potential (EPSP) that was reversibly blocked by the cholinergic antagonists d-tubocurarine, atropine and mecamylamine indicating that the afferent-to-PPR synapse used ACh as a neurotransmitter and that functional nAChRs and mAChRs exist in the *Manduca* CNS. α -Btx suppressed the EPSP but only at micromolar concentrations. The authors have attributed this ineffective blocking ability to either an α -btx insensitive nAChR or the presence of a penetration barrier that was not permeable to α -btx. The EPSP was depolarised in response to nicotine but demonstrated 60-fold lower sensitivity when compared with literature data for the *Periplaneta* motoneurone, Df (David and Sattelle, 1984). However, responses evoked from other cholinergic agonists did not show such a distinctive difference (Table 1.1), implying that the relative insensitivity to nicotine was due to a selective protective mechanism(s).

| Agonist | <i>Manduca</i> † | <i>Periplaneta</i> * | Ratio |
|-----------|------------------|----------------------|-------|
| Nicotine | 91 | 1.5 | 61 |
| Carbachol | 117 | 200 | 0.59 |
| TMA | 13800 | 950 | 14.5 |

Table 1.1. ED₅₀ values (μM; the concentration producing an half-maximal response) for cholinergic agonists on the PPR motoneuron from *Manduca* and the Df motoneuron from *Periplaneta*. *David and Sattelle, 1984 (Values estimated from the dose response curves shown in Figure 1 of this reference) †Trimmer and Weeks, 1989.

The inability of the *Manduca* CNS to respond to nicotine with the same degree as a nicotine sensitive insect indicates the existence of several mechanisms that play either a major or minor role in defending the CNS against the toxic effects of nicotine.

Metabolism in the central nervous system

Although the midgut appears to be the major site of nicotine metabolism in *Manduca*, Morris (1983a) has shown that the CNS can also metabolise nicotine. In insects, the nerve cord is highly heterogeneous, comprising neurons, glia, branches of the tracheal system and some adherent fat body cells. Of these, it was found that nicotine metabolism occurred in the axonal/glial and the cell body/neuropile/glial regions in addition to the trachea. Comparison with the the nicotine sensitive cockroach, *Periplaneta*, demonstrated that the CNS of both insects metabolised nicotine but the latter to a lesser extent. This has since been confirmed by Snyder *et al.* (1993) who demonstrated that nerve cords from *Manduca* larvae were able to metabolise nicotine to nicotine-1-N-oxide, probably by cytochrome P-450. Unlike midgut metabolism however, CNS metabolism was not inducible. Morris (1983a) postulated that a nicotine detoxifying mechanism could only protect the CNS if the rate of nicotine metabolism at least equalled the rate of penetration, otherwise equilibration of nicotine between the haemolymph and the extracellular space would happen. This would explain why the CNS connectives and the tracheal cells were able to metabolise nicotine: if nicotine were not detoxified, it would be free to diffuse from the CNS connectives and tracheal cells into the neuropile of the ganglion. It was speculated that the peripheral layers of the insect CNS play a detoxifying role similar to that of the vertebrate liver. This would be necessary as the insect gut is very permeable and consequently ingested food can enter the haemolymph of the general body cavity, a space adjacent to the blood-brain barrier,

in contrast to the vertebrate system whereby ingested food passes via the hepatic portal system to the liver before meeting the blood-brain barrier.

Related studies have shown that clearance of nicotine and its metabolites from the CNS of *Manduca* is different from that of the nicotine sensitive *Periplaneta* (Morris, 1983b). In fact, the kinetics of nicotine and metabolite efflux in *Manduca* seem slower and less complicated than that of *Periplaneta*, indicating a greater ability in *Manduca* CNS to immobilise nicotine. In contrast, organic molecules such as leucine and sucrose show similar efflux patterns for both *Manduca* and *Periplaneta* nerve cords (Morris, 1983c), implying that only nicotine and its metabolites are handled differently in the two insect species studied.

Barriers to nicotine penetration

It is possible that the blood-brain barrier in *Manduca* could result in some resistance to nicotine: this would require prevention of the diffusion of nicotine in both its hydrophilic protonated form and its lipophilic unprotonated form. The lipophilic unprotonated form can cross membranes such as the ion barrier and blood-brain barrier but only the hydrophilic protonated form can interact with the nAChR (Yamamoto *et al.*, 1995). However, lipophilic nicotine once through the blood-brain barrier can establish a new equilibrium, resulting in the presence of protonated nicotine at the synapse. In *Manduca* and *Periplaneta*, the blood-brain barrier is at the level of the perineurium (Lane *et al.*, 1977). Electron micrographs have shown that larval *Manduca* ganglia appear to be surrounded by basal cells of the perineurium which form an epithelium with very few cell to cell contacts thus minimising the overall area available for intercellular diffusion of protonated nicotine (Morris and Harrison, 1984). Hence, accessible sites for the intercellular diffusion of nicotine into the neuropile are distinctly limited, directly contrasting with the nicotine sensitive *Periplaneta* whose basal perineurial cell layer is much richer in cell to cell contacts (Lane *et al.*, 1977). Transcellular penetration through the cytoplasm of unprotonated nicotine appears to be prevented by enzymatic metabolism by the CNS (see Metabolism in the CNS above) as ultrastructural examination shows the presence of smooth endoplasmic reticulum associated with cytochrome P-450 in the perineurium (Morris and Harrison, 1984).

The presence of a tracheo-glial barrier which could substitute for the blood-brain barrier in those regions penetrated by branches of the tracheal system has also been postulated (Lane *et al.*, 1977; Morris and Harrison, 1984). It is thought that as the tracheal cells within the CNS contain smooth endoplasmic reticulum, they may also possess

detoxifying cytochrome P-450 enzymes which prevent nicotine from harming the nervous system.

Selective transport

The inclusion of alkaloid pumps within the CNS, which would transfer nicotine out of the extracellular space adjacent to the nicotine receptive membrane and into the cytoplasm could also account for the nicotine insensitivity seen in *Manduca*. However, measurements of nicotine uptake have suggested that there is no difference between nicotine penetration of nerve cords for *Manduca* and the nicotine sensitive *Periplaneta* (Morris, 1983a). Furthermore, utilisation of a cationic alkaloid pump inhibitor, N'methylnicotinamide (NMN) did not decrease the rate of nicotine uptake in *Manduca* nerve cords. Global extracellular electrophysiological recordings on *Manduca* nerve cords demonstrated that simultaneous application of 10-fold excess NMN with 0.25mM nicotine evoked neural activity (Morris, 1984). However, this potentiating effect could be due to a synergistic action of NMN and nicotine at the nAChR. Moreover, Trimmer and Weeks (1989) have reported that electrophysiological recordings on an isolated motoneuron from *Manduca* show only a weak potentiation of the nicotinic response with application of NMN, its main effect being to increase the rate of response and to reduce the rate of recovery, suggesting that the presence of alkaloid pumps does not markedly contribute to the nicotine resistance of the motoneuron. In fact, the continued (although diminished) resistance to nicotine after NMN treatment and during the prepupal developmental stage when cationic pumps were inactive suggested that active exclusion was not the major defence mechanism against nicotine.

Receptor modification

The relative insensitivity seen toward nicotine by the CNS even after desheathing of the neuron suggests that the nicotine resistance may be conferred, at least in part, by a modified receptor. Morris (1984) and Trimmer and Weeks (1989) have both postulated that an alteration of the insect nAChR that is genetically based could be achieved by the production of a novel receptor which would give rise to the unusual pharmacological characteristics seen in *Manduca* neurons. Alternatively, post-translational modifications of an otherwise typical insect nAChR could result in the CNS nicotine insensitivity. Either way, modifications such as these may have arisen throughout evolution to accommodate an alkaloid-rich diet and now remain as an intrinsic attribute of *Manduca*.

Recently, studies on the mutant, dieldrin-resistant γ -aminobutyric acid subtype A (GABA_A) receptor, of the fruit fly, *Drosophila melanogaster* (RDL) have shown that a single naturally occurring amino acid substitution (A302S) in the proposed channel lining

domain (French-Constant *et al.*, 1993a) confers resistance to the insecticides dieldrin (a cyclodiene) and picrotoxinin, both in heterologously expressed homomeric RDL GABA_A receptors and *in situ* GABA_A receptors of *Drosophila* (French-Constant *et al.*, 1993b; Zhang *et al.*, 1994). It has been postulated that the change of a methyl group of the alanine side chain to an aliphatic hydroxyl group on the serine side chain results in an alteration of the binding site in the channel pore thus hindering ion channel block and/or the mechanism(s) whereby these insecticides reduce GABA induced responses. Furthermore, this mutation also reduces the potency of the structurally distinct phenylpyrazole insecticide, fipronil (Hosie *et al.*, 1995). This suggests that the single amino acid substitution in this mutant GABA_A receptor results in a non-selective mechanism for protection against various insecticides.

Notably, there is evidence to suggest that different subtypes of the nAChR exist in nicotine resistant and wild type *Drosophila* derived from the Hikone-R strain (Hall *et al.*, 1978). It was established that the nicotine resistant type HR created a shift in the isoelectric point of an α -btx binding component, indicating that the mutation affected receptor structure. Both the nicotine resistance factor and the pI were X-linked, suggesting that the HR locus encoded a structural polypeptide in the α -btx binding complex (Hall, 1980).

Hence, the existence of a modified insect nAChR where substitution of an amino acid that would typically be used in binding nicotine is replaced by an amino acid that is incapable of nicotine binding could result in the nicotine insensitivity found in *Manduca*. In the *Drosophila* cases outlined above, resistance is a developed ability within the *Drosophila* species to tolerate doses of toxicants which would prove lethal to most individuals in a normal population of the same *Drosophila* species (reviewed in Oppenoorth, 1985). Resistance to nicotine in *Manduca* larvae is somewhat different, being an intrinsic trait seen in all offspring (Trimmer and Weeks, 1989). Consequently, a modified nAChR that prevented the binding of nicotine and ultimately a "depolarising blockade" in *Manduca* larvae could be termed a natural mutant.

There have been no published studies indicating functional nAChRs by electrophysiological recordings on adult *Manduca* cholinoreceptive neurons. Given Maddrell and Gardiner's (1976) finding that protective transport mechanisms existed in the larval but not in the adult form of *Manduca*, both larval and adult forms should be examined for the presence of a modified nAChR.

Summary

In summary, a number of protective mechanisms could account for the nicotine insensitivity seen in *Manduca*. These are listed in Table 1.2.

| REGION | MECHANISM |
|----------------------------|--|
| Midgut and digestive tract | Rapid metabolism of nicotine [•] Excretion of nicotine metabolites [•] |
| CNS | Metabolism of nicotine [■] Barriers against nicotine penetration to the nAChR [♦] Selective transport [○] |
| nAChR | Modified and unable to interact with nicotine [○] |

Table 1.2. Potential protective mechanisms against nicotine in *Manduca*. [•] Snyder *et al.*, 1994; [■] Morris, 1983a, Snyder *et al.*, 1993; [♦] Morris and Harrison, 1984; [○] Morris, 1984, Trimmer and Weeks, 1989.

The nAChR and the possibility of a mutated receptor in *Manduca* are considered in greater detail for the remainder of this chapter. Vertebrate nAChRs have been intensively studied and therefore are considered before the lesser known insect nAChRs.

1.2 Vertebrate nAChRs

1.2.1 The Ligand Gated Ion Channel Family

AChRs are divided into two classes on the basis of their pharmacological properties: those activated by the mushroom alkaloid, muscarine are termed muscarinic AChRs (mAChRs) and those activated by the tobacco alkaloid, nicotine are termed nicotinic AChRs (nAChRs). Both types span the lipid membranes of nerve and muscle. However, mAChRs are metabotropic receptors, in which the binding of ACh causes mAChRs to interact with G proteins sequentially, modulating various effector molecules such as ion channels and enzymes and thus eliciting a response, while nAChRs are ligand gated ion channels (LGICs), receptors that contain an integral hydrophilic ion channel through which particular ions can traverse the membrane.

Several other neurotransmitter receptors belong to the LGIC family. In vertebrates, ACh, 5HT₃ and glutamate gate cation channels typically found at excitatory synapses whereas glycine and GABA_A gate anion channels typically found at inhibitory synapses. Amino acid sequence analysis has determined that the ACh, 5HT₃, GABA_A and glycine receptors are homologous whereas excitatory glutamate receptors share no overall homology with the other neurotransmitter receptors but do between themselves, thus resulting in two families of LGICs. More recently, ATP has been found to gate excitatory ion channels (termed P_{2X} receptors), thus resembling the ACh LGIC family functionally. However, sequence analysis predicts a structure resembling the family that form inwardly rectifying and pH-sensitive K⁺ channels and amiloride sensitive Na⁺ channels and therefore cannot be classified as belonging to either family (Brake *et al.*, 1994). The nAChR is subdivided into muscle and neuronal type receptors which share some common structural and functional features.

1.2.2 Muscle-type nAChRs

Muscle-type receptors and in particular those of the electric ray, *Torpedo* are the prototype of LGICs. They are introduced here and discussed in greater detail in Chapter 5, where muscle nAChRs from the mouse were expressed in *Xenopus* oocytes as a training exercise in two electrode voltage clamp recording.

Release of ACh into the synaptic cleft of the neuromuscular junction results in the activation of muscle-type nAChRs and muscle fibre contraction. Purification and characterisation of these muscle-type nAChRs was assisted by the finding that the

muscle derived electroplax of the electric eel, *Electrophorus*, and the electric ray, *Torpedo*, were rich in nAChRs that were almost irreversibly bound by the *Bungarus multicinctus* snake venom toxin, α -bungarotoxin (α -btx), that could be radiolabelled to high specific activity (reviewed in Chiappinelli, 1993).

Analysis of the ~270 kDa purified receptor by denaturing polyacrylamide gel electrophoresis resulted in four bands, α , β , γ and δ of apparent molecular weights 38, 50, 59 and 64 kDa respectively, that were assembled into an heteromeric pentamer with a resulting stoichiometry of $(\alpha)_2\beta\gamma\delta$ (Lindstrom *et al.*, 1979). Each α subunit housed one binding site for ACh, giving two ACh binding sites per receptor. Purified receptors, reconstituted in lipid bilayers resulted in functional ion channels (reviewed in McNamee and Ochoa, 1982), as did expression in *Xenopus* oocytes of the combination $\alpha\beta\gamma\delta$ (Mishina *et al.*, 1984; also see Chapter 5). Developmental changes affect the subunit composition of the muscle-type receptor with the γ subunit of the embryonic form being replaced by an ϵ subunit in the adult form. This in turn alters the functional properties of the receptor (Mishina *et al.*, 1986).

1.2.3 Conserved structural features of the nAChR

Microsequencing of the peptides revealed high homology between subunits from the same species (for *Torpedo* see Noda *et al.*, 1982, 1983a,b) and of the same subunit between different species. Sequence data such as these has allowed the identification of the following conserved features for nAChR subunits:

- an extracellular N-terminal region which in the α subunit contains the agonist and competitive antagonist binding sites
- four hydrophobic regions designated as putative transmembrane domains (TM1-4) of which TM2 is proposed to line the ion channel and contain non-competitive antagonist binding sites
- a variable intracellular or cytoplasmic loop between TM3 and TM4, incorporating putative phosphorylation sites
- a short extracellular C-terminal domain.

All nAChR subunits are characterised by a conserved sequence in the N-terminal extracellular region corresponding to a cysteine loop from residues 128 to 142 in the *Torpedo* α subunit (see Appendix 1). In addition, α subunits are characterised by the presence of a pair of disulphide bridged cysteines at positions 192 and 193 (*Torpedo* numbering) in the extracellular region. α Subunits are referred to as the major agonist

binding subunit although adjacent non- α subunits are thought to play a part. These basic features are shown in a diagrammatic model of the nAChR, Figure 1.3. The contribution of various residues in particular subunits to the formation of the ACh binding site, the α -btx binding site and the ion channel is further discussed in section 1.2.7.

1.2.4 Neuronal-type nAChRs

Cloning of genes and cDNAs encoding neuronal nAChRs

Within the last decade, rapid progress has been made in the isolation of neuronal nAChRs through the use of complementary DNA (cDNA) probes. There are two types of neuronal receptor subunits designated α and β (or non- α). In agreement with muscle-type receptor subunits, the α subunits are defined by the presence of two contiguous cysteines located at positions 192 and 193 in *Torpedo* (see Appendix 1). Although neuronal non- α subunits are named β subunits for clarity, this does not imply that they are necessarily equivalent to the muscle-type β subunits. The majority of putative nAChR encoding genes have been isolated from chick and rat neural tissues. Currently, seven putative α subunits ($\alpha 2$ - $\alpha 8$) and three putative β subunits ($\beta 2$ - $\beta 4$) have been cloned from neurons (muscle type receptors are deemed to possess $\alpha 1$ and $\beta 1$ subunits), although $\alpha 8$ has been isolated only from chick and $\alpha 6$ only from rat (reviewed in Sargent, 1993). A further subunit, $\alpha 9$, has been located in certain endocrine and sensory organ cells of the rat (Elgoyhen *et al.*, 1994). Table 1.3 reveals that overall amino acid homology between cDNAs encoding neuronal and muscle-type α subunits from the same species is 35-55%. The same applies to β subunits (Sargent, 1993). Currently, there is no agreement on the evolution of vertebrate neural and muscle subunits. Of the most recent studies, Ortells and Lunt (1995) postulate that muscle $\alpha 1$ and non- α subunits have evolved from two different groups of neural α -like and non- α subunits, respectively whereas le Novère and Changeux (1995) have speculated that the muscle $\alpha 1$ subunit evolved alongside the vertebrate neural receptors while the muscle non- α subunits were an independently evolving group. The homology increases on comparison of the transmembrane domains and parts of the extracellular region and decreases on comparison of the cytoplasmic loop between TM3 and TM4, indicating variability specific to a particular subunit (Sargent, 1993). Furthermore, high homology is obtained from comparison of corresponding sequences in chick and rat. The neuronal α subunits themselves subdivide into two or three classes depending on their ability to bind α -btx: $\alpha 7$ and $\alpha 8$ are highly homologous to each other but distinct from other, α -btx insensitive, neuronal subunits. The $\alpha 9$ subunit is distinct not only from the α -btx

insensitive subunits but also from $\alpha 7$ and $\alpha 8$ subunits (Schoepfer *et al.*, 1990; Elgoyhen *et al.*, 1994).

| | C α 1 | r α 1 | C α 2 | r α 2 | C α 3 | r α 3 | C α 4 | r α 4 | C α 5 | r α 5 | r α 6 | C α 7 | r α 7 | C α 8 | r α 9 |
|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| C α 1 | 100 | 84 | 48 | 47 | 52 | 50 | 48 | 49 | 45 | 46 | 48 | 38 | 37 | 38 | 36 |
| r α 1 | | 100 | 48 | 47 | 53 | 52 | 49 | 51 | 44 | 45 | 47 | 37 | 37 | 35 | 36 |
| C α 2 | | | 100 | 75 | 58 | 59 | 68 | 65 | 53 | 53 | 53 | 40 | 39 | 38 | 36 |
| r α 2 | | | | 100 | 57 | 57 | 67 | 65 | 52 | 52 | 52 | 40 | 40 | 39 | 34 |
| C α 3 | | | | | 100 | 81 | 55 | 57 | 50 | 50 | 62 | 39 | 39 | 37 | 34 |
| r α 3 | | | | | | 100 | 56 | 58 | 49 | 49 | 61 | 39 | 38 | 36 | 36 |
| C α 4 | | | | | | | 100 | 72 | 53 | 51 | 53 | 38 | 38 | 38 | 34 |
| r α 4 | | | | | | | | 100 | 54 | 53 | 54 | 37 | 37 | 36 | 34 |
| C α 5 | | | | | | | | | 100 | 82 | 49 | 37 | 37 | 36 | 35 |
| r α 5 | | | | | | | | | | 100 | 49 | 36 | 37 | 35 | 35 |
| r α 6 | | | | | | | | | | | 100 | 37 | 36 | 36 | 36 |
| C α 7 | | | | | | | | | | | | 100 | 87 | 69 | 36 |
| r α 7 | | | | | | | | | | | | | 100 | 68 | 35 |
| C α 8 | | | | | | | | | | | | | | 100 | 35 |
| r α 9 | | | | | | | | | | | | | | | 100 |

Table 1.3. Amino acid identities between cDNA encoding muscle and neuronal α subunits of chick (c) and rat (r). Identities calculated from an alignment of α subunits using the gcg program Distances (uncorrected) (Swofford and Olsen, 1990). SwissProt accession numbers were: C α 1 p09479; r α 1 p25108; C α 2 p09480; r α 2 p12389; C α 3 p09481; r α 3 p094757; C α 4 p09482; r α 4 p09483; C α 5 p26152; r α 5 p20420; r α 6 p43143; C α 7 p22770; r α 7 q05941; r α 9 p43144.

1.2.5 Confirmation of function in neuronal nAChRs

Confirmation that these genes encode functional nAChRs has been elicited from purification of brain nAChRs that are encoded by these genes, expression of functional nAChRs from combinations of these genes in non-neuronal cells and expression of these genes in autonomic neurons and in brain nuclei from which nicotinic responses have been obtained.

Purification of neuronal nAChRs

nAChRs were immunopurified from chick brain by affinity chromatography with a monoclonal antibody (MAb) to electric organ nAChR. This resulting preparation

contained two bands, of 49 and 59 kDa, on a denaturing gel (Whiting and Lindström, 1986a). Another MAb against this immunopurified material, immunoprecipitated a third band at 75 kDa, as well as the two previously isolated. Using MAbs, two types of nAChR were distinguished, one containing the 49 and 59 kDa peptides and the other containing the 49 and 75 kDa peptides (Whiting *et al.*, 1987a; Whiting and Lindstrom, 1987a). Protein sequencing determined that the 75 kDa peptide corresponded to the sequence predicted for the $\alpha 4$ subunit (Whiting *et al.*, 1991) whilst the 49 kDa peptide corresponded to the sequence predicted for the $\beta 2$ subunit (Schoepfer *et al.*, 1988). It was thought that the 59 kDa peptide may contain both $\alpha 2$ and trace amounts of $\alpha 3$ (discussed in Sargent, 1993). Therefore, chick brain appeared to comprise nAChRs composed of $\alpha 4/\beta 2$ and $\alpha 2/\beta 2$ subunits and maybe $\alpha 3/\beta 2$ subunits. More recently, it was demonstrated that the MAb to electric organ nAChR recognised the $\alpha 5$ subunit and could be assembled with $\alpha 4$, thus suggesting that some nAChRs in chick brain comprised $\alpha 4$, $\alpha 5$ and $\beta 2$ subunits (Conroy *et al.*, 1992).

Whiting and Lindstrom (1987b) also immunopurified nAChRs from rat brain using a MAb against the chick 49 kDa peptide. This resulted in two bands of 51 and 79 kDa which were found to correspond to the sequences predicted for $\beta 2$ and $\alpha 4$ subunits respectively (Whiting *et al.*, 1987b; Schoepfer *et al.*, 1988).

Using subunit specific MAbs, it has been shown that chick ciliary ganglion neurons contain three nAChR subtypes. The first subtype contains $\alpha 3$, $\alpha 5$ and $\beta 4$ subunits (Vernallis *et al.*, 1993). About 20% of these type receptors also contain the $\beta 2$ subunit, suggesting that neuronal nAChRs have the potential to be as complex as the muscle-type receptor. The final subtype contains the $\alpha 7$ gene product and does not appear to be associated with the subunits found in the two other subtypes of receptor (Conroy and Berg, 1995).

Finally, α -btx sensitive nAChRs have been purified from vertebrate brain using α -btx affinity columns and have revealed between one to five bands on denaturing gels in the range 45 to 72 kDa (Norman *et al.*, 1982; Conti-Tronconi *et al.*, 1985; Kemp *et al.*, 1985; Whiting and Lindstrom, 1987b; Gotti *et al.*, 1991), suggesting either the presence of an homomeric or an heteromeric receptor. Schoepfer *et al.* (1990) demonstrated that the majority of α -btx sensitive nAChRs in chick brain contained the $\alpha 7$ and/or $\alpha 8$ subunits (see Chapter 3, section 3.1.1).

Functional expression of neuronal nAChR genes

Expression studies using *Xenopus* oocytes (see Chapter 5 for further explanation of this technique) have demonstrated that the genes thought to encode nAChRs do actually represent functional nAChRs. Boulter *et al.* (1987), Wada *et al.* (1988) and Deneris *et al.* (1989a) showed that oocytes expressing either cRNA encoding rat $\alpha 2$, $\alpha 3$ or $\alpha 4$ subunits in combination with cRNA encoding the rat $\beta 2$ subunit formed functional heteromeric receptors that were activated by ACh and nicotine. ACh evoked responses were not blocked by α -btx in all three combinations. In contrast, only $\alpha 3/\beta 2$ and $\alpha 4/\beta 2$ were blocked by neuronal bungarotoxin (n-btx). Alone, only injection of $\alpha 4$ produced functional responses but these were only small depolarisations at high concentrations of ACh in one third of the trials. Similarly, ACh evoked currents were obtained from cDNAs encoding chick $\alpha 3/\beta 2$, $\alpha 3/\beta 4$, $\alpha 4/\beta 2$ and $\alpha 4/\beta 4$ subunit combinations (Couturier *et al.*, 1990a). Both α -btx and n-btx had little effect on the $\alpha 4/\beta 2$ combination, though other combinations were not tested (Ballivet *et al.*, 1988; Bertrand *et al.*, 1990). Moreover, no subunit alone produced functional responses (Couturier *et al.*, 1990a).

Neither the rat $\alpha 5$ cRNA nor the chick $\alpha 5$ cDNA resulted in the formation of functional nAChRs when co-injected with cRNA encoding rat $\beta 2$, $\beta 3$ or $\beta 4$ subunits (Boulter *et al.*, 1990) or cDNA encoding chick $\beta 2$ or $\beta 4$ subunits (Couturier *et al.*, 1990a). Combinations of the rat $\alpha 6$ gene with various β subunits and the rat $\beta 3$ gene with several α subunits also did not result in functional nAChRs (Deneris *et al.*, 1989b; Sargent, 1993). Recently however, it was established that chick $\alpha 5$ mRNA could form functional nAChRs when co-injected with mRNA encoding the $\alpha 4$ and $\beta 2$ subunits, producing responses that differed significantly from oocytes injected with just $\alpha 4$ and $\beta 2$ mRNA (Ramirez-Latorre *et al.*, 1996). Interestingly, it has been suggested that the $\alpha 5$ subunit has been misnamed (Conroy *et al.*, 1992). As $\alpha 5$ lacks one or two of the tyrosines thought to play a role in ligand binding: tyrosine 190 in chick and tyrosines 93 and 190 in rat (Couturier *et al.*, 1990a; Boulter *et al.*, 1990 respectively; see section 1.2.7), it is possible that $\alpha 5$ represents a β type and therefore structural subunit of the nAChR. However, it does contain the vicinal cysteines that define the α subunit. This may set a precedent for *Manduca*, which quite possibly lacks the tyrosine residues similarly to the $\alpha 5$ subunit and therefore may not form a functional receptor.

Thus, it appears that some functional nAChRs are formed from the combination of specific α and β subunits giving an α/β heteromeric receptor. However, this heteromeric model has been complicated by the demonstration of functional receptors from α subunits alone.

Using *Xenopus* oocytes, it was shown that cDNA encoding the chick $\alpha 7$ subunit alone, in the absence of any co-injected β subunit cDNA, led to the assembly of functional neuronal nAChRs (Couturier *et al.* 1990b). These were more sensitive to nicotine than ACh, desensitised very rapidly and showed significant voltage dependence at elevated membrane potentials. Furthermore, the $\alpha 7$ channel was completely blocked by nanomolar concentrations of α -btx. Co-injection of cDNAs encoding $\alpha 7$ with either $\beta 2$, $\beta 3$ or $\beta 4$ subunits, did not result in functional responses differing from those obtained with $\alpha 7$ alone, indicating that $\alpha 7$ did not co-assemble with any of these subunits into a functional heteromeric nAChR. However, it was suggested that *in vivo*, the $\alpha 7$ subunit was perhaps part of an heteromeric α -btx sensitive nAChR. This has also been indicated by pharmacological comparison using inhibition of radiolabelled α -btx binding between native $\alpha 7$ containing nAChRs from chick brain and homomeric chick $\alpha 7$ nAChRs extracted from *Xenopus* oocytes (Anand *et al.*, 1993). The slight differences in affinity for particular ligands (especially cytisine, a nicotinic agonist that differentiates between β subunits - see Functional diversity of expressed nAChRs below) suggested that the native $\alpha 7$ nAChRs probably did not exist as homomers but were combined with other subunits.

The cDNA encoding another subunit, $\alpha 8$ (Schoepfer *et al.*, 1990) has also been shown to form functional homomeric nAChRs in *Xenopus* oocytes (Gotti *et al.*, 1994). The channels were blocked by nanomolar concentrations of α -btx but unlike $\alpha 7$ showed equal affinity for ACh and nicotine. Interestingly, co-injection of $\alpha 7$ and $\alpha 8$ in varying ratios, always resulted in responses typical of the $\alpha 7$ subtype, although the $\alpha 7/\alpha 8$ combination occurs *in vivo* (see above; Schoepfer *et al.*, 1990; Gotti *et al.*, 1994; Chapter 3, section 3.1.1).

In contrast to $\alpha 7$ and $\alpha 8$ homomeric receptors, the cRNA encoding $\alpha 9$ receptors formed functional homomeric receptors that were activated by ACh but not by nicotine although nicotine reduced the currents evoked by ACh (Elgoyhen *et al.*, 1994). In addition, co-injection of $\alpha 9$ with either $\beta 2$ or $\beta 4$ did not result in the formation of channels that were activated by nicotine. However, both the nicotinic agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP) and the muscarinic agonist, oxotremorine M induced inward currents in $\alpha 9$ injected oocytes while ACh evoked responses were blocked by nanomolar concentrations of α -btx as well as d-tubocurarine (a nicotinic antagonist) and atropine (a muscarinic antagonist). Thus cRNA encoding the $\alpha 9$ subunit was able to form functional homomeric receptors with an unusual mixed nicotinic-muscarinic pharmacology.

Functional diversity of expressed nAChRs

Pharmacological profiles of various subunit combinations demonstrated that particular sensitivities to agonists were peculiar to distinct combinations. Using combinations of cRNA encoding rat $\alpha 2$, $\alpha 3$ and $\alpha 4$ with $\beta 2$ and $\beta 4$, Luetje and Patrick (1991) revealed that those combinations containing the $\beta 2$ subunit were almost completely unresponsive to cytosine compared with ACh whereas those combinations containing the $\beta 4$ subunit were more sensitive to cytosine than any other agonist tested. Moreover, Papke and Heinemann (1993) established that cytosine was a partial agonist at $\beta 2$ subunit containing receptors. For example, cytosine bound with high affinity to $\alpha 4\beta 2$ receptors but had low agonist efficacy being relatively ineffective at opening the ion channel. It is pertinent to suggest that a similar mechanism may exist in *Manduca*, where nicotine might bind to the nAChR with high affinity but has poor efficacy as an agonist, thus enabling *Manduca* to survive nicotine ingestion (see Chapter 3, section 3.5). Furthermore, replacing the α subunit combined with $\beta 2$ resulted in different orders of sensitivity to nicotine and ACh (Luetje and Patrick, 1991). Similarly, different sensitivities to ACh were found with chick combinations of $\alpha 3$, $\alpha 4$, $\beta 2$ and $\beta 4$ (Couturier *et al.*, 1990a). Experiments with antagonists also demonstrated that the potency of n-btx and lophotoxin analog-1 depended on which α subunit was combined with rat $\beta 2$, for example, $\alpha 3/\beta 2$ showed the greatest sensitivity to n-btx (Luetje *et al.*, 1990). Summarised, these results indicated that both α and β subunits contributed to the pharmacological diversity of neuronal nicotinic receptors. In addition, the distinctive agonist sensitivities could serve as a means of distinguishing different neuronal subtypes *in vivo*.

Subunit composition has been shown to affect single channel conductances. Papke *et al.* (1989) showed that different combinations of $\alpha 2$, $\alpha 3$ or $\alpha 4$ mRNA with $\beta 2$ mRNA resulted in receptors expressed in *Xenopus* oocytes with unique single channel properties. Two conductance states were observed for $\alpha 2/\beta 2$ and $\alpha 3/\beta 2$ indicating the presence of two distinct nAChR populations following the injection of a single α subunit and a single β subunit. Multiple conductance states were also observed in $\beta 4$ containing nAChRs which differed from those containing $\beta 2$ subunits (Papke and Heinemann, 1991).

This phenomenon may be due to either indiscriminate subunit assembly or different post translational modifications in the oocyte. In the former case this may be due to mixing in stoichiometry (for example $2\alpha 2\beta$, $3\alpha 2\beta$, $2\alpha 3\beta$, $3\alpha 3\beta$) or mixing in subunit arrangements around the ion channel (for example $\alpha\beta\alpha\beta\beta$ or $\alpha\alpha\beta\beta\beta$). However, [^{35}S]methionine labelling of mRNAs encoding $\alpha 4$ and $\beta 2$ subunits gave a ratio of 1:1.46 implying a

pentamer containing two α and three β subunits (Anand *et al.*, 1991), as did channel conductance studies on putative ion channel mutated subunits (Cooper *et al.*, 1991). Therefore, an α : β stoichiometry of 2:3 is favoured as this correlates with a size (~300 kDa) consistent with a pentameric structure, as shown in Figure 1.4.

Correlating subunit composition to native receptors

At present, there is little evidence to identify native nAChRs with those subunit combinations expressed in *Xenopus* oocytes. Only in the rat interpeduncular nucleus (IPN) where it is known that the genes encoding $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\beta 2$ are expressed, does the channel conductance of 35pS (Mulle *et al.*, 1991) correspond to the largest conductance state obtained from the $\alpha 2/\beta 2$ combination in *Xenopus* oocytes (34pS; Papke *et al.*, 1989), suggesting that a composition of $\alpha 2/\beta 2$ may form the main class of nAChRs on IPN neuronal cell bodies. However, comparison of agonist potency does not agree with this conclusion as cytisine and ACh are equally effective at eliciting responses on IPN neurons (Mulle *et al.*, 1991) but not on $\beta 2$ containing nAChRs expressed in *Xenopus* oocytes (Luetjje and Patrick, 1991).

1.2.6 Presynaptic nAChRs

Although nAChRs mediate fast excitatory transmission at the neuromuscular junction and in autonomic ganglia of the PNS, there is scant evidence for a similar role of nAChRs in the vertebrate CNS. It has been suggested that the majority of nAChRs in the CNS are presynaptic (residing at nerve terminals rather than cell bodies) and play a modulatory role in the brain (Sargent, 1993; Wonnacott, 1996). Those presynaptic nAChR most extensively studied are found on striatal dopamine terminals. It has been demonstrated that nicotine at low concentrations (EC_{50} : submicromolar) elicited [3H]dopamine release in superfused synaptosome preparations (Clarke and Reuben, 1996). Partial agonism by cytisine (see section 1.2.5, Functional diversity of expressed nAChRs above) on the same preparation and lesion studies on dopaminergic terminals suggest that this presynaptic nAChR correlates with the $\alpha 4/\beta 2$ subunit combination (reviewed in Wonnacott, 1996).

Another example of presynaptic nAChRs transpires from the ability of low concentrations of nicotine (EC_{50} : 170nM) to enhance synaptic transmission of glutamate in the medial habenula (McGehee *et al.*, 1995). The nicotinic response was blocked by α -btx, suggesting the involvement of the $\alpha 7$ subunit and this was confirmed by antisense knockdown experiments. Interestingly, nicotine was still able to enhance glutamate

transmission (that was now insensitive to α -btx), suggesting the presence of an heteromeric $\alpha 7$ receptor (see section 1.2.5, Functional expression of neuronal nAChR genes).

1.2.7 Agonist and antagonist binding sites

Given the hypothesis that the nicotine insensitivity in *Manduca* is due to a naturally mutated receptor (see section 1.1.3, Receptor modification), it is important to discuss the structural features of the nAChR that may contribute to the nicotine binding site. Although the vertebrate nAChR (especially the *Torpedo* receptor) has been used for the analysis of residues important for binding agonists and lining the ion channel, a similar structure incorporating particular residues is presumed to exist for insect nAChRs.

Pharmacological characterisation of nAChRs in muscle and nerve tissue has resulted in the identification of pharmacologically discrete receptor populations. Three classes of ligands have been used (reviewed in Chiappinelli, 1993):

- 1) Agonists such as ACh, nicotine and epibatidine which elicit opening of the channel.
- 2) Competitive antagonists such as α -btx, d-tubocurarine and dihydro- β -erythroidine which prevent opening of the channel by binding to a site(s) overlapping the cholinergic binding site.
- 3) Non-competitive antagonists such as histrionicotoxin which either sterically block ion flux by binding to the ion channel or prevent channel opening by binding to regulatory sites distinct from the ACh binding site.

Localisation of binding sites

Of these ligands, radiolabelled nicotine and α -btx have been the most utilised in determining high affinity binding sites for different subtypes of native neuronal nAChRs (see Chapter 3). Mapping of high affinity binding sites in rat brain has determined that [3 H]nicotine and [125 I] α -btx sites are distinct from each other with [125 I] α -btx sites being distributed most densely in the cerebral cortex, hypothalamus, hippocampus, inferior colliculus and in some nuclei of the brain stem whereas [3 H]nicotine sites were mostly located in the thalamic nuclei (except posterior and intralaminar nuclei), interpeduncular nucleus, medial habenula and the superior colliculus while binding was also prominent in the substantia nigra pars compacta and ventral tegmental area, the molecular layer of the dentate gyrus, the presubiculum and the cerebral cortex (Clarke *et al.*, 1985).

The [^3H]nicotine binding site locations correlate with those observed from immunostaining using MAbs against the $\beta 2$ subunit (Swanson *et al.*, 1987). In chick, MAbs against $\alpha 7$ and $\alpha 8$ subunits have shown different distributions of immunoreactivity compared with a MAb against the $\beta 2$ subunit (Britto *et al.*, 1992).

In addition, the pattern of gene expression for particular subunits has been useful in defining those populations of nAChRs on different neurons. *In situ* hybridisation assays have shown that each gene is expressed in a distinct pattern. For example, high levels of $\alpha 7$ transcripts have been located in the olfactory areas, the amygdala, the hippocampus, the hypothalamus and the cerebral cortex of the rat, areas that overlap with high affinity [^{125}I] α -btx binding sites (Seguela *et al.*, 1993), while rat $\alpha 2$ transcripts have limited distribution, primarily in the interpeduncular nucleus (Deneris *et al.*, 1989a) where some of the [^3H]nicotine sites are located.

Structural features of the nAChR

Given that the agonist binding sites and the ion channel are so topographically distinct, an allosteric mechanism of action which uses multiple interconvertible conformations (resting, active, intermediate and desensitised) has been proposed (Changeux *et al.*, 1984). As yet, how the binding of agonists activates ion channel opening has not been demonstrated. Recently however, electron images of postsynaptic membranes from *Torpedo* have delineated the structures for the open and closed channel forms of the receptor (Unwin, 1995). Briefly, it appears that the binding of ACh initiates small rotations of the subunits in the extracellular region, which elicit a change in configuration of the α -helices lining the ion channel, pulling away the ion channel gate and thus allowing ion flow through the pore.

This leads onto the importance of particular residues in forming attachment points for ligand binding and in forming the ion channel gate.

Structure of the ACh binding site

The topology of the ACh binding sites on *Torpedo* nAChR has been investigated using photoaffinity labelling probes. The interaction of these probes with the nAChR suggest that many residues of the α subunit contribute to the ACh binding site. Those residues of particular importance are shown in Figure 1.3.

Initial studies identified the disulphide linked vicinal cysteines at positions 192 and 193 (*Torpedo* numbering), when reduced, as the site of incorporation of the affinity label MBTA (4-(N-maleimido)-benzyltrimethylammonium) (Kao *et al.*, 1984). Further

investigation using the photoaffinity label DDF (p-(N,N-dimethylamino) benzenediazonium fluoroborate) which in the dark behaves as a reversible competitive antagonist of the nAChR but irreversibly blocks the binding of cholinergic ligands following irradiation at 295nm, has led to a three loop model of the agonist and competitive antagonist binding site (Dennis *et al.*, 1986, 1988; Galzi *et al.*, 1990). In this, DDF identifies the residues tryptophan 86 and tyrosine 93 in loop A, the residues tryptophan 149 and tyrosine 151 in loop B and the residues tyrosine 190, the vicinal cysteines 192 and 193 and tyrosine 198 in loop C. The three loop model is supported not only by the fact that, within these three loops DDF labelling is competitive with α -btx and carbamylcholine but also by the labelling of tyrosine 190 by an analog of the competitive antagonist, lophotoxin (Abramson *et al.*, 1989) and by the equilibrium labelling of tyrosine 190, cysteine 192 and tyrosine 198 by [³H]nicotine, the latter residue being labelled the most predominantly (Middleton and Cohen, 1991).

The residues labelled by DDF are conserved at homologous positions in all known α subunits from muscle and neuronal nAChRs in all species examined to date, excluding the $\alpha 5$ subunit which lacks the residues tyrosine 93 and tyrosine 190 in rat (Boulter *et al.*, 1990) and tyrosine 190 in chick (Couturier *et al.*, 1990a). Moreover, muscle type non- α subunits lack those amino acids labelled by DDF whereas neuronal β subunits contain tryptophan 149 and tyrosine 93, suggesting that these β subunits play a minor role in binding (reviewed in Devillers-Thiéry *et al.*, 1993). Thus in *Manduca*, it might be expected that one or more of these residues are altered, thus preventing agonist binding (see section 1.2.5, Functional expression of neuronal nAChR genes).

The cysteine loop

The cysteine residues 128 and 142 (*Torpedo* numbering) have been shown to form a disulphide bridge in the α subunit. This stretch of residues is commonly termed the 'cys loop' and occurs in a region that is highly conserved between the α , β , γ and δ subunits, suggesting that the cysteines at either end of the cys loop are disulphide cross-linked in each subunit (Kao and Karlin, 1986). Computer modelling has suggested that the cys loop forms a rigid amphiphilic β -hairpin that is a major determinant of the binding site (Cockcroft *et al.*, 1990). It was postulated that the invariant aspartate at position 11 of the cys loop is the anionic site interacting with the positively charged amine group of agonists (i.e. the cationic headgroup); that the proline residue at position 9 induces the turn of the cys loop and interacts with the agonist at its electronegative centre and that differences in the type of residue at position 6 resulted in selective recognition of a particular agonist. However, there is no experimental evidence to support such claims

a)

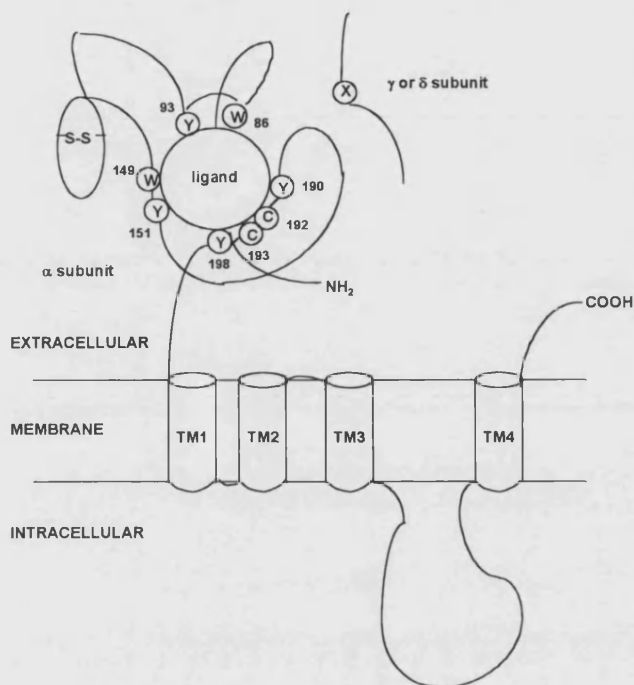
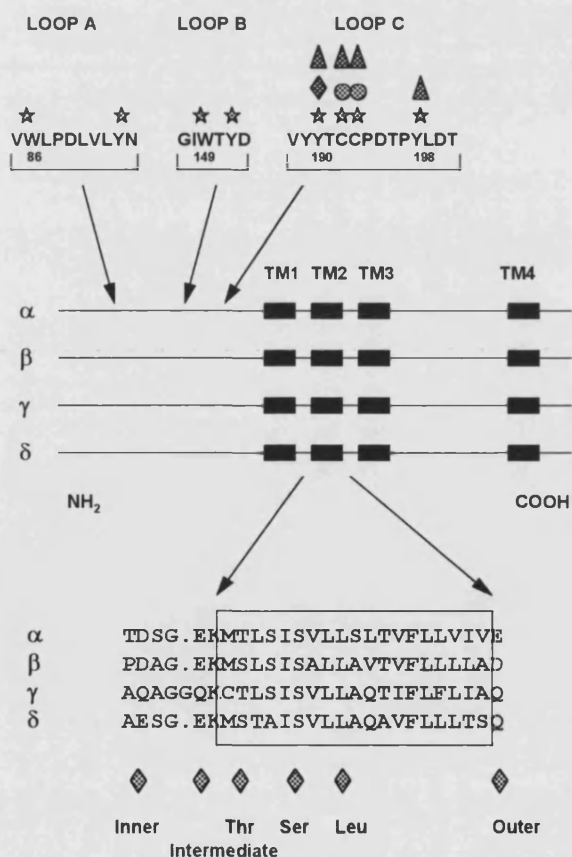


Figure 1.3. Structure of the nAChR.

a) Stylised model of the α -subunit of the nAChR indicating residues involved in ligand binding in *Torpedo*.

b)



b) Amino acids involved in ligand binding or formation of ion channel as determined by affinity labelling and/or site directed mutagenesis on *Torpedo* $\alpha 1$, $\beta 1$, γ or δ subunits. Upper: residues labelled by DDF (\star), MBTA (\bullet), lophotoxin (\blacklozenge) and nicotine (\blacktriangle). Middle: line diagram of location of transmembrane domains (not to scale). Lower: location of rings (\blacklozenge) postulated to line the ion channel. See text for further explanation. Redrawn from Changeux, 1990.

although the cys loop has been implicated in the binding of allosteric effectors such as physostigmine (see Non-competitive agonists below; Chapter 2, section 2.5.3).

Functional appearance of the ACh binding site

The functional significance of the photoaffinity labelled residues has been investigated by site directed mutagenesis. In *Torpedo* nAChR, substitution of the vicinal cysteines at positions 192 and 193 with serines abolishes the response to ACh (Mishina *et al.*, 1985), indicating that cysteines 192 and 193 are imperative for ACh binding.

In separate experiments, three aromatic residues in the chick $\alpha 7$ subunit, namely tyrosine 92, tryptophan 148 and tyrosine 187 corresponding to the *Torpedo* residues tyrosine 93, tryptophan 149 and tyrosine 190 that were labelled by DDF, were mutated to phenylalanine (Galzi *et al.*, 1991). These substitutions caused a decrease in the apparent affinity for the agonists ACh and nicotine. The affinity for ACh was decreased 10-fold by the mutations Y92F and Y187F and 100-fold by the mutation W148F, while the affinity for nicotine was decreased by different amounts: 2-fold for the mutations Y92F and Y187F and 300-fold for the mutation W148F, indicating that ACh and nicotine make different interactions with these three residues. In particular, tryptophan 148 appears to be important for nicotine binding and it may be that this residue is missing in a modified receptor in *Manduca*. In addition, affinity for the competitive antagonist, dihydro- β -erythroidine was decreased by 4-fold for Y92F and Y187F and 30-fold for W148F and to a lesser extent for α -btx. Other properties investigated, such as the voltage dependency of the ion response were not changed, indicating that the mutations selectively affected the recognition of cholinergic ligands by the receptor protein.

Further studies where tyrosine 93, 190 and 198 in the mouse muscle α subunit have been individually replaced with serine show a much larger decrease in affinity for ACh compared with substitution by other aromatic amino acids (Aylwin and White, 1994). For example, the mutations Y198F and Y198W decreased affinity 5-fold whereas the mutation Y198S resulted in a 30-fold decrease in affinity, suggesting that at position 198, an interaction of the ligand with an aromatic side chain occurs. Moreover, experiments on the replacement of tryptophan 54 of the chick $\alpha 7$ receptor by phenylalanine, alanine or histidine residues have resulted in a progressive decrease in binding affinity and responses evoked by ACh and nicotine (Corringer *et al.*, 1995). Approximately, a 2-fold, 10-fold and 50-fold reduction in EC_{50} values was seen on W54F, W54A and W54H mutations respectively, suggesting once again the importance of aromatic residues. That tryptophan 54 was not included in the three loop model, indicated that it formed a complementary component of the binding site.

That aromatic residues play an important role in agonist binding within the nAChR, is similar to the situation in AChE, where atomic resolution of the structure has shown that the substrate binding site is in an 'aromatic gorge' (Sussman *et al.*, 1991). It has been proposed that the electronegative character of the photoaffinity labelled aromatic residues is sufficient to associate with the diffuse positive charge of the quaternary ammonium ions representing the cationic head of ACh (reviewed in Devillers-Thiéry *et al.*, 1993). Relating this to *Manduca*, it is conceivable that these important aromatic residues have been replaced with non-aromatic residues and therefore do not bind nicotine efficiently.

Contribution of non- α subunits to the ACh binding site

In muscle, each nAChR contains two α subunits and therefore two ACh binding sites. For effective activation of the receptor, the co-operative binding of two molecules of ACh (one to each binding site) is required and a variety of evidence has indicated that these sites are not equivalent (reviewed in Devillers-Thiéry *et al.*, 1993) and that this was probably due to contributions to the binding site made by non- α subunits. Indeed, the γ subunit was specifically photoaffinity labelled by DDF (Langenbuch-Cachat *et al.*, 1988). Moreover, α and γ subunits and α and δ subunits were photoaffinity labelled with [^3H]d-tubocurarine (Pederson and Cohen, 1990). The α - γ subunit pair formed the high affinity d-tubocurarine binding site whilst the α - δ subunit pair formed the low affinity site. As the $\beta 1$ subunit did not contribute to the binding site, it was suggested that the subunits were arranged in a rosette of $\alpha\gamma\alpha\beta\delta$ enabling rotational pseudosymmetry around the channel pore, as shown in Figure 1.4.

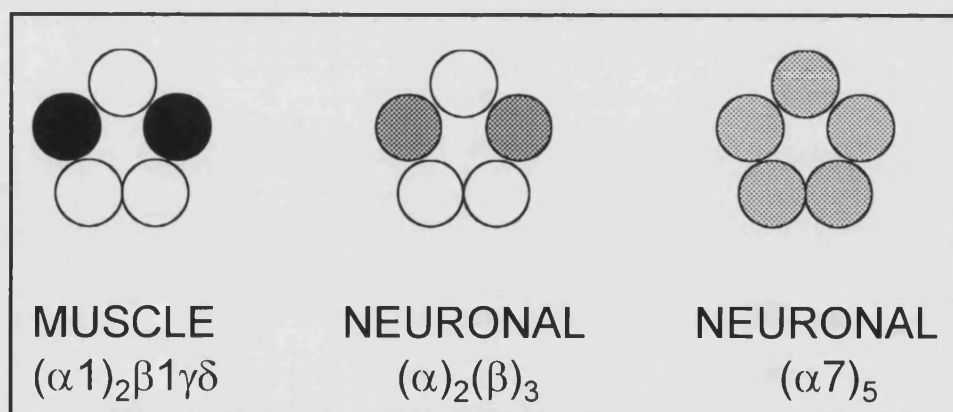


Figure 1.4. Proposed subunit arrangement around the ion channel for nAChRs expressed in *Xenopus* oocytes. Muscle arrangement: $\alpha\gamma\alpha\beta\delta$ (Pederson and Cohen, 1990); neuronal arrangement either heteromeric: $\alpha\beta\alpha\beta\beta$ or homomeric: $\alpha\alpha\alpha\alpha\alpha$ (Sargent, 1993).

It appears that the non- α subunits may help to create a negatively charged subsite of anionic residues which interact with the ligand. Czajkowski and Karlin (1991) demonstrated that the bifunctional affinity reagent, S-(2-[3 H]-glycylaminoethyl) dithio-2-pyridine (containing sulphydryl and carboxyl interacting groups) labelled the negatively charged carboxyl groups from aspartate and glutamate residues specifically in a region in the *Torpedo* δ subunit. Replacement in the δ subunit of the aspartate and glutamate residues at positions 180 and 189 with asparagine and glutamine residues respectively resulted in a decrease in the apparent affinity for ACh (Czajkowski *et al.*, 1993). As these residues were conserved in the γ , δ and ϵ subunits of all species, but not in β subunits it was suggested that they formed a negative subsite at both the α - γ and α - δ ACh binding sites.

On the other hand, there is abundant proof that the neuronal $\beta 2$ and $\beta 4$ subunits play a role in ligand binding as pharmacological profiles of combinations of subunits differ according to the β subunit used (Luetjje and Patrick, 1991; see section 1.2.5, Functional diversity of expressed nAChRs). Investigations with chimeric models composed of $\beta 2/\beta 4$ hybrids coexpressed with $\alpha 4$ subunits in *Xenopus* oocytes showed that the β subunits agonist or competitive antagonist selectivity were due to interactions with the N-terminal domain, particularly residues 1 to 80 (Wheeler *et al.*, 1993).

Non-competitive agonists

Physostigmine, an AChE inhibitor, has been shown to have both an agonistic and channel blocking effect on the nAChR (Shaw *et al.*, 1985). Activation of the nAChR reconstituted in membrane vesicles by physostigmine was insensitive to α -btx but was inhibited by the physostigmine-competitive MAb, FK1 (Okonjo *et al.*, 1991). Similarly, Storch *et al.* (1995) demonstrated that FK1 did not affect channel activation by ACh but did inhibit channel activation by physostigmine in PC12 cells, suggesting that physostigmine acted via binding sites distinct from ACh. Using the dominant prototope of FK1, it was established that physostigmine bound to the residues 118-137 of the α subunit in *Torpedo* (Schröder *et al.*, 1994), a region that contained lysine 125, the amino acid photoaffinity labelled by [3 H]physostigmine (Schrattenholz *et al.*, 1993), a residue not labelled by ACh binding site affinity labels. This region overlaps the cys loop (see section 1.2.7, The cysteine loop above). These data indicated that nAChR activation by a second pathway that may function physiologically as an allosteric control of receptor activity was possible.

The α -Bungarotoxin binding site

α -Btx has been used as a major determinant in the classification of different nAChR subtypes as it is a potent antagonist of muscle $\alpha 1$ subunits (reviewed in Chiappinelli, 1993) and neuronal $\alpha 7$ and $\alpha 8$ subunits as well as $\alpha 9$ subunits (Couturier *et al.*, 1990b; Schoepfer *et al.*, 1990; Elgoyhen *et al.*, 1994). α -Btx with a molecular weight of ~ 8 kDa, is significantly larger than any of the agonists that activate the nAChR, therefore it would be expected that there is some overlap with the ACh binding site. The importance of the α subunit was initially established by demonstration of α -btx binding to a denatured $\alpha 1$ subunit from *Torpedo* (Haggerty and Froehner, 1981). However, considering the selective nature of α -btx binding, the contribution of specific domains to formation of the α -btx binding site must be necessary.

Several regions of the α subunit have been implicated in the binding site of α -btx. Using synthetic peptides, it has been established that the region $\alpha 181$ -200 in *Torpedo* is imperative for α -btx binding (Conti-Tronconi *et al.*, 1990). Homologous regions are found in muscle $\alpha 1$ from chick (positions 179-198) and neuronal $\alpha 7$ and $\alpha 8$ from chick (positions 181-200) which, as synthetic peptides, specifically bind α -btx with differing affinities (McLane *et al.*, 1991a). Approximately the same segment ($\alpha 183$ -204) was shown to bind α -btx in bacterial fusion proteins from a variety of organisms ranging from human to *Drosophila* (Ohana and Gershoni, 1990).

Reduction and alkylation of the peptides corresponding to the *Torpedo* α subunit sequence 181-200 from a variety of organisms has resulted in a reduction of α -btx binding activity, although oxidation of the same sequence has little effect (Conti-Tronconi *et al.*, 1990; McLane *et al.*, 1991a,b). This has suggested that although oxidation of the adjacent cysteines to form a vicinal disulphide bond may not be critical for α -btx binding, one or both cysteines are involved in forming the interface of this region with α -btx as cysteine alkylation leads to reduced α -btx binding.

Other regions shown to complex with α -btx using synthetic peptides based on the *Torpedo* sequence include, $\alpha 55$ -74 (Conti-Tronconi *et al.*, 1990) and also regions $\alpha 1$ -10, $\alpha 32$ -41, $\alpha 100$ -115 and $\alpha 122$ -150 (Mulac-Jericevic and Atassi, 1987a,b), indicating that a complex interaction with several parts of the molecule takes place.

Detailed analysis has revealed that particular residues, distinct from those labelled by photoaffinity reagents delineating the agonist binding site are essential for α -btx interaction with the nAChR. Using site directed mutagenesis, Mishina *et al.* (1985)

demonstrated that the cysteine residues at $\alpha 128$ and $\alpha 142$ (in *Torpedo*) were crucial residues for α -btx binding. It appeared that these mutations affected the conformation of the α subunit or its assembly with the other subunits as substantially smaller amounts of the α , γ and δ subunits were recovered by electrophoretic analysis on the *Xenopus* oocytes expressing the mutants. Conti-Tronconi *et al.* (1991) analysed the $\alpha 181$ -200 region of *Torpedo* by employing non-conservative glycine substitutions to each residue within this peptide segment. It was found that valine 188, tyrosine 189, tyrosine 190, cysteines 192 and 193 and proline 194 were imperative for α -btx binding. To a lesser degree, the residues tryptophan 184, lysine 185, tryptophan 187, aspartate 195, threonine 196, proline 197 and tyrosine 198 appeared influential on α -btx binding. A similar investigation on the peptide 180-199 of the rat $\alpha 5$ sequence indicated that lysine 184, arginine 187, cysteine 191 and proline 195 were essential for binding of α -btx (McLane *et al.*, 1991c).

Several organisms possess muscle type nAChRs that, although activated by cholinergic ligands, do not bind α -btx. Sequence analysis of the muscle α subunit from a species of elapid snake resistant to α -btx revealed high homology with other muscle α subunits. However, within the region of the ligand binding site, four major substitutions were found: at position 184 a tryptophan was replaced with a phenylalanine, at position 185 a lysine was replaced with a tryptophan, at position 187 a tryptophan was replaced with a serine and at position 194 a proline was replaced with a leucine (Neumann *et al.*, 1989), as well as several other substitutions. Further analysis using single or multiple mutations in fragments of the α subunit corresponding to the region 185-196 that were cloned and expressed in bacteria, have indicated that proline residues at positions 194 and 197 and aromatic amino acids at positions 187 and 189 of the α subunit are required to confer α -btx sensitivity, as changes at these positions to non-proline and non-aromatic residues respectively result in α -btx resistance (Barchan *et al.*, 1995; Kachalsky *et al.*, 1995).

X-ray crystallographic analysis has revealed that α -btx (and also cobra-toxin, the 'long' toxins) comprises a globular head, the folding of which is determined by four disulphide bridges, and three loops hanging down from the head arranged side by side in a β -sheet configuration (Walkinshaw *et al.*, 1980; Love and Stroud, 1986). It has been suggested that a large area of the α -btx molecule formed by three sequence segments, namely both sides of the central loop and the lower tip of the third loop are involved in nAChR interactions (Chatrenet *et al.*, 1990). A correspondingly large area on the nAChR, formed by several residues within the sequence segments $\alpha 181$ -200 and $\alpha 55$ -74 could make contact with the α -btx molecule (Conti-Tronconi *et al.*, 1991). Amalgamating the

data described here from various sources suggests that the residues forming attachment points with the toxin include tyrosine (or other aromatic residue) 189, cysteine 192 and 193 and proline 194 and 197, as shown in Figure 1.5.

| | 180 | 200 |
|---|---|-----|
| α -Btx sensitive α subunits | | |
| <i>Torpedo</i> α 1 | DYRGWKHWV <u>YYT</u> <u>CCP</u> DT <u>P</u> YLD | |
| Chick α 1 | EARGWKHWV <u>YYA</u> <u>CCP</u> DT <u>P</u> YLD | |
| Chick α 7 | GIPGKRTE <u>S</u> <u>F</u> Y <u>E</u> <u>CC</u> KE. <u>P</u> YPD | |
| α -Btx insensitive α subunits | | |
| Chick α 2 | NAIGRYNSKKYD <u>CC</u> TE. <u>I</u> YPD | |
| Snake α 1 | DYRGFWHVSVNYS <u>CC</u> LD <u>T</u> <u>P</u> YLD | |
| Mongoose α 1 | EARGWKHNVTY <u>A</u> <u>CC</u> LT <u>T</u> HYLD | |

Figure 1.5. Sequence alignment of α 180-200 from α -btx sensitive and α -btx insensitive subunits, indicating residues postulated to form interactions with α -btx (underlined, bold amino acids). *Torpedo* α 1: Noda *et al.*, 1982; Chick α 1, α 2: Nef *et al.*, 1988; Chick α 7: Couturier *et al.*, 1990b; Schoepfer *et al.*, 1990; Snake α 1: Neumann *et al.*, 1989; Mongoose α 1: Barchan *et al.*, 1992.

As discussed in section 1.1.2, *Manduca* brain appears to be rich in [125 I] α -btx binding sites. Therefore, [125 I] α -btx will prove a useful tool in defining the binding affinity of agonists for this site. This is the subject of Chapter 3.

The ion channel

The permeability response resulting from the activation of nAChRs can be inhibited by non-competitive blockers (NCBs) such as histrionicotoxin, chlorpromazine and QX222. They operate by entering the open channel and binding to a site located within it, consequently decreasing the amplitude of the ion flux. Accordingly, many NCBs are voltage dependent. Separate from the agonist binding site, there appear to be two classes of NCB binding site: an high affinity histrionicotoxin sensitive site within the ion channel and 10-30 low affinity, histrionicotoxin insensitive sites thought to be located at the lipid-protein interface (reviewed in Devillers-Thi  ry *et al.*, 1993).

Affinity labelling using [^3H]chlorpromazine showed that it was incorporated into the four different subunits of *Torpedo* (Heidemann and Changeux, 1984) indicating that the high affinity binding site for the NCBs was located in the axis of pseudosymmetry of the molecule within the ion channel. Extensive photoaffinity labelling studies with [^3H]chlorpromazine indicated that those residues labelled were common to the TM2 (Giraudat *et al.*, 1986, 1987, 1989; Revah *et al.*, 1990) and thus a model of the ion channel was established. In this, TM2 segments of each subunit line the ion channel in a symmetrical arrangement with these segments probably forming an α helical structure. Site directed mutagenesis has confirmed that TM2 lines the ion channel. Homologous residues from each subunit, lining the ion channel lumen form five superimposed rings: the Threonine, Serine, equatorial Leucine, Valine and outer Leucine rings from N to C terminal ends of the TM2 segment (reviewed in Devillers-Thiéry *et al.*, 1993). Additional rings of negatively charged residues (the Inner and Intermediate rings at the N-terminal end and the Outer ring at the synaptic cleft) are found at the extremes of the lumen (Imoto *et al.*, 1988). These are shown in Figure 1.3.

Functional expression studies of nAChRs mutated at these rings suggest the presence of a stratified organisation within the ion channel (reviewed in Bertrand *et al.*, 1993). Briefly, it appears that the Serine, equatorial Leucine, Valine and outer Leucine rings control ion channel gating whilst others such as the Threonine ring control ion size selectivity. Interestingly, mutations in the Serine ring alter monovalent cation selectivity whereas mutations in the outer Leucine ring modify monovalent versus divalent ion selection and mutations between the inner and intermediate rings of negatively charged residues control cation versus anion selectivity. This organisation indicates that ion channel access is highly regulated.

Phosphorylation of the nAChR

The phenomenon of desensitisation at the nAChR is well documented. However, its role is unclear as at the synapse, AChE will typically remove ACh at a rate sufficient to prevent induction of desensitisation. Therefore, desensitisation must be either physiologically important or the result of a mechanism in which the nAChR structure relaxes as the initial low affinity ACh binding becomes high affinity as the binding site refolds to accommodate the bound ligand (Stroud *et al.*, 1990). It is possible that phosphorylation regulates the extent of desensitisation of the receptor. For example, phosphorylation of purified, reconstituted *Torpedo* nAChR by cAMP-dependent protein kinase was correlated with acceleration of the rapid phase of agonist induced desensitisation by an *in vitro* flux assay (Huganir *et al.*, 1986). Furthermore, increasing the number of tyrosine residues phosphorylated by protein tyrosine kinase in purified

nAChR reconstituted in lipid bilayers resulted in acceleration of the rate of desensitisation, indicating that the responsive state of the nAChR was regulated by the extent and type of phosphorylated residues (Hopfield *et al.*, 1988). Indeed, it has been postulated that the nAChR autoregulates its own functional activity by a novel signal transduction pathway, whereby the nAChR is a substrate for an uncharacterised protein kinase *in situ* and that activity of this protein kinase is stimulated by calcium ions that permeate through the activated nAChR ion channel (Miles *et al.*, 1994). More recently, site directed mutagenesis studies have shown that mutant receptors lacking phosphorylation sites are expressed and assembled normally but desensitise at a significantly slower rate than wild type nAChR (Hoffman *et al.*, 1994). Interestingly, phosphorylation has also been implicated in subunit assembly (Green *et al.*, 1991).

1.3 Insect nAChRs

ACh is thought to be the major excitatory neurotransmitter in the CNS of insects due to the presence of components of cholinergic synapses such as the ACh synthesising enzyme, ChAT, the degrading enzyme, AChE and binding sites for the nicotinic cholinergic ligand, α -btx in a variety of insects, such as *Manduca* (see section 1.1.2; reviewed in Colhoun, 1963; Pichon and Manarache, 1985; Restifo and White, 1990). In contrast to vertebrates, insect neuromuscular transmission appears to be glutamatergic (Usherwood, 1980). Hence, there does not appear to be a synonymous muscle-type nAChR in insects. Information regarding insect nAChRs is scarce, those best characterised being nAChRs from *Drosophila*, the two locust species, *Schistocerca gregaria* (desert locust) and *Locusta migratoria* (migratory locust) and the cockroach, *Periplaneta*.

1.3.1 Binding sites for nicotinic cholinergic ligands in the insect CNS

Pharmacological characterisation

α -Btx, a potent antagonist of vertebrate neuromuscular and neuronal $\alpha 7$ - $\alpha 8$ receptors, and also $\alpha 9$ receptors, has been a useful pharmacological tool in the study of putative nAChRs in insects. High affinity binding sites for [125 I] α -btx, with a dissociation constant (K_d) in the range 0.1-2nM and an abundance (B_{max}) of between 0.2 and 1.4 pmol/mg membrane protein have been identified by several groups using either centrifugal assays or filter binding assays on membrane extracts from *Drosophila* heads (Dudai, 1977, 1978; Dudai and Amsterdam, 1977; Schmidt-Nielsen *et al.*, 1977; Rudloff, 1978). A pharmacological profile typical for nAChRs is displayed by the binding of [125 I] α -btx: it is specifically displaced with high affinity in pre-incubation experiments by other nicotinic ligands such as unlabelled ACh, nicotine, α -btx and d-tubocurarine and is less sensitive to muscarinic ligands such as atropine and quinuclidinyl benzilate (QNB) (see Schmidt-Nielsen *et al.*, 1977 for examples of dose response curves). More recently, Scatchard analysis has demonstrated the presence of at least two different classes of high affinity [125 I] α -btx binding sites in *Drosophila* head and embryonic membranes, with K_d values of ~ 0.1 nM (class 1) and ~ 4 nM (class 2) and maximal binding of ~ 0.24 and ~ 1.1 pmol/mg protein respectively (Schloss *et al.*, 1988).

An α -btx binding component has also been demonstrated for several other insects. For *Schistocerca*, K_d values between 0.8 and 1.7nM, with a B_{max} of 1.2pmol/mg have been obtained for [125 I] α -btx binding (Filbin *et al.*, 1983; MacAllan *et al.*, 1988). Again, a

pharmacological profile typical of an nAChR was seen, specific [125 I] α -btx binding was displaced by unlabelled ACh, nicotine, α -btx and d-tubocurarine, as well as the extremely potent nicotinic agonist and antagonist, anatoxin-a and methyllycaconitine (MLA) respectively. A nicotinic profile was also seen for the closely related species, *Locusta* where unusually, nicotine displaced [125 I] α -btx binding with considerably higher affinity than ACh (Breer, 1981). [125 I] α -Btx binding sites exhibiting nicotinic pharmacology have also been reported for the house fly, *Musca domestica* (Harris *et al.*, 1979; Mansour *et al.*, 1980; Jones *et al.*, 1981), the honey bee, *Apis mellifera* (Tomizawa *et al.*, 1995a) as well as *Periplaneta* (Lummis and Sattelle, 1985) and *Manduca* (Sanes *et al.*, 1977). The latter two insects are interesting as they enable direct comparison between ligand binding studies and electrophysiological experiments on identifiable neurons, and therefore may show that α -btx is binding to a functional nAChR. It is notable that there have been few studies with other radioligands that show specific binding to the nAChR in insects. Most recently, Tomizawa *et al.* (1995a) demonstrated that [3 H]phencyclidine (PCP) bound to *Apis* membranes at high and low affinity sites and demonstrated typical nicotinic pharmacology. It was suggested that these high and low affinity sites corresponded to the insect equivalent of the vertebrate NCB site on the ion channel and the ACh binding site respectively (see section 1.2.7).

Biochemical characterisation and reconstitution into lipid bilayers

Purification of the α -btx binding components from several insects has resulted in the isolation of a putative nAChR. Hall (1980) increased the purity of the α -btx binding component from *Drosophila* heads 1200-fold by passing Triton X-100 solubilised membrane extracts over a cobratoxin-Sepharose 4B affinity column, eluting the adsorbed material with carbamylcholine, re-applying to a second cobratoxin-Sephadex affinity column where contaminating material was removed by high salt solution before finally eluting with carbamylcholine. Treatment of *Drosophila* head membranes with sodium deoxycholate at pH9.0, followed by affinity chromatography was also successful (Rudloff *et al.*, 1980). These solubilised α -btx binding complexes were demonstrated by gel filtration chromatography to have a molecular weight of 460,000 - 500,000 (Hall, 1980; Rudloff *et al.*, 1980; Schmidt-Glenewinkel *et al.*, 1981) and by sucrose density gradients to have a molecular weight of 250,000-300,000 (Hall, 1980; Jimenez and Rudloff, 1980). The two different classes of α -btx binding sites could not be separated by sucrose density centrifugation, ion exchange chromatography or affinity chromatography on lentil-lectin agarose (Schloss *et al.*, 1991). Four subunits have been identified on SDS-polyacrylamide gel electrophoresis, with molecular weights of 78, 64, 57 and 48 kDa (Schmidt-Glenewinkel *et al.*, 1981).

Purification of membranes from *Musca* heads has identified two subunits of ~40 and ~20 kDa (Osborne *et al.*, 1982) whilst three subunits of 60, 41 and 25 kDa have been isolated from partially purified *Schistocerca* supraoesophageal ganglion membranes (Filbin *et al.*, 1983). In contrast an α -btx binding component from the related species, *Locusta* has been purified to homogeneity and migrates as a 250-300 kDa complex in native polyacrylamide gels and as a single 65 kDa band under denaturing conditions (Breer *et al.*, 1985). The isolated protein was reconstituted in a planar lipid bilayer where it formed ion channels that were activated by cholinergic agonists and blocked by d-tubocurarine when subjected to patch-clamp analysis (Hanke and Breer, 1986), thus indicating that an α -btx sensitive protein from *Locusta* was a functional homomeric nAChR.

Autoradiographic localisation

Several autoradiographic studies using [125 I] α -btx have been carried out on frozen sections of *Drosophila*. Given that in insect brain a distinction exists between the ganglion cell bodies which are located in the cortex at the periphery of the brain and the neuropile which houses the inner parts of the brain including the synapses that are axodendritic rather than axosomatic (Dudai and Amsterdam, 1977) it was important to discover that toxin binding was confined to the synaptic neuropile areas of the *Drosophila* CNS in adult heads (Dudai and Amsterdam, 1977; Schmidt-Nielsen *et al.*, 1977; Rudloff, 1978) and could be abolished by pre-incubation of sections with nicotine, d-tubocurarine or unlabelled α -btx, strongly implying that [125 I] α -btx was labelling nAChRs. Rudloff (1978) also located [125 I] α -btx binding to the neuropile in *Drosophila* larvae and pupae. Only the lamina of the optic lobes showed no detectable [125 I] α -btx binding.

Autoradiographic localisation of [125 I] α -btx on the sixth abdominal ganglion of *Periplaneta* again revealed dense binding in the central neuropile either side of the midline that could be completely removed by pre-incubation with nicotine or d-tubocurarine (Sattelle *et al.*, 1983). As this was the region of the ganglion where cercal afferent fibres made their synaptic contacts with the dendritic branches of the giant interneurons, it suggested that the toxin was labelling a nAChR. However, dense binding was also observed in the periphery of the ganglion, a non-synaptic region occupied by glial cells and neuronal cell bodies and was only partially removed on pre-treatment with d-tubocurarine indicating that some of this [125 I] α -btx binding was non-specific.

Finally, [125 I] α -btx binding to frozen sections of *Manduca* brain has been demonstrated (Hildebrand *et al.*, 1979; see section 1.1.2). The anatomical organisation of the antennal

lobes facilitates the histological distinction between synaptic regions, nonsynaptic regions and neuronal cell bodies. In each antennal lobe, sensory axons from the ipsilateral antennae enter the neuropile via the antennal nerve and end in the glomeruli. These glomeruli contain the primary afferent synapses and specialised cellular components associated with them. Hildebrand *et al.* (1979) showed that toxin binding was heavily concentrated in the glomeruli and therefore associated with putative nAChRs.

Electrophysiological characterisation

The presence of functional nAChRs on identified neurons in insects has been demonstrated by electrophysiological recordings from afferent terminals of sensory neurons that make synaptic contact with interneurons and motoneurons. Single afferents generate EPSPs on stimulation by mechanical or electrical means that can be modulated by application of drug.

Sattelle *et al.* (1983) have investigated synaptic transmission between cercal sensory neurons and the giant interneuron 2 (GI2) in the terminal (sixth) abdominal ganglion of *Periplaneta*. GI2 was a convenient preparation as it facilitated direct comparison of the pharmacology between synaptic and extrasynaptic nAChRs on the same neuron. It was demonstrated that nanomolar concentrations (50nM) of α -btx (comparatively close to the K_d of 5nM established from binding studies - see Lummis and Sattelle, 1985) irreversibly blocked the cercal afferent input to GI2. Furthermore, the time taken to block the EPSP was increased under conditions which either enhanced presynaptic release of ACh (increased frequency of stimulation or inclusion of 4-aminopyridine - a potassium channel blocker known to increase the duration of presynaptic action potential in *Periplaneta* (Hue *et al.*, 1976)) or inhibited the hydrolysis of ACh within the synapse (inclusion of physostigmine, an AChE inhibitor). In contrast, application of QNB (a muscarinic agent) was ineffective at concentrations up to 1 μ M (compared with a K_d of 8nM in binding experiments - see Lummis and Sattelle, 1985). Similar results were shown with the cell body of GI2, it being sensitive to ionophoretically applied ACh and blocked by α -btx at lower concentrations than QNB. Thus it was established that both synaptic and extrasynaptic membranes of GI2 contained α -btx sensitive nAChRs.

Further nAChRs have been identified in *Periplaneta* on the fast coxal depressor motoneurone, Df which is located on the metathoracic ganglion. David and Sattelle (1984) obtained dose response curves for four ionophoretically applied agonists. Nicotine was the most effective, followed by ACh, carbamylcholine and TMA. The ACh depolarisation could be blocked by nanomolar concentrations of α -btx but only by high

micromolar concentrations of QNB. Under voltage clamp conditions, it was established that α -btx block was voltage-independent compared with d-tubocurarine block which was voltage dependent.

The presence of nAChRs in the grasshopper, *Schistocerca nitens* has also been revealed (Goodman and Spitzer, 1979). Ionophoretic application of ACh onto the dorsal unpaired median (DUM) neurons resulted in depolarisation of the membrane that was abolished by removal of Na⁺ ions and reduced by d-tubocurarine. Nicotine also depolarised the membrane whereas α -btx failed to block the ACh response at micromolar concentrations, an observation also seen for DUM neurons of the metathoracic ganglion in *Periplaneta* (Harrow *et al.*, 1982) indicating the presence of α -btx insensitive nAChRs. In this context, it is interesting that Lees *et al.* (1983) observed two populations of nAChRs in cultured neurons from the CNS of embryonic *Periplaneta*, the majority of which were sensitive to α -btx and a minority that were insensitive to α -btx.

Voltage clamp experiments on isolated, thoracic ganglion neuronal somata from *Locusta* indicate the presence of nAChRs due to a rapid depolarisation in response to ACh, nicotine, anabasine and TMA and inhibition by α -btx (Benson, 1992).

Electrophysiological characterisation of *Drosophila* has been limited. Recently, Albert and Lingle (1993) have shown that single channel recordings in cultured *Drosophila* neurons established the presence of nAChRs as ACh and nicotine both evoked responses that were blocked by α -btx.

Functional nAChRs have also been identified in *Manduca* using the planta hair afferent-to-motoneuron synapse (Trimmer and Weeks, 1989) as discussed in section 1.1.3.

1.3.2 Cloning of genes and cDNAs that encode insect nAChRs

nAChRs from vertebrates and invertebrates have the same topology and therefore the subunits that have been isolated from insects have been divided into α -like and β -like subunits: all contain the N-terminal extracellular region housing the cysteine loop, the four transmembrane domains, the cytoplasmic loop between TM3 and TM4 and the extracellular C-terminal tail but only the α -like subunits contain the vicinal cysteines.

The gene of the first α -like subunit ALS, from *Drosophila* was identified through cross-hybridisation with a fragment of the chick $\alpha 2$ gene on a *Drosophila* genomic library and

the corresponding cDNA was isolated by screening a *Drosophila* cDNA library with restriction fragments of the ALS gene (Bossy *et al.*, 1988). Utilisation of probes from the ALS cDNA has isolated two further α -like subunit encoding genes, *Drosophila* α -like subunit 2, D α 2 (Jonas *et al.*, 1990) and *Drosophila* α -like subunit 3, D α 3 (unpublished observations cited in Gundelfinger, 1992). The corresponding cDNA of D α 2 has been isolated through the same means (Baumann *et al.*, 1990) and also by probing a cDNA library with a mixture of synthetic oligonucleotides deduced from a highly conserved stretch of ten amino acids preceding TM4 (Sawruk *et al.*, 1990a). The latter cDNA clone is commonly referred to as SAD, the second α -like subunit from *Drosophila*. The cDNA encoding the γ subunit of the nAChR from *Torpedo* was used to isolate firstly the cDNA and secondly the gene for the first β -like subunit of the AChR, ARD, from *Drosophila*, (Hermans-Borgmeyer *et al.*, 1986; Sawruk *et al.*, 1988). The gene encoding the ARD subunit was also isolated at the same time by another group, again by cross-hybridisation with cDNA encoding the Torpedo γ subunit and named the AChR64B protein (Wadsworth *et al.*, 1988). The cDNA encoding a second β -like subunit from *Drosophila* (SBD; Sawruk *et al.*, 1990b) was isolated in the same manner as adopted by Sawruk *et al.* (1990a).

An α -like and a β -like subunit have been isolated from *Schistocerca gregaria*. The gene encoding a β -like subunit was isolated by cross hybridisation with a chick β 2 subunit (Marshall *et al.*, 1988) and a restriction fragment of this clone was then used to isolate the cDNA encoding an α -like subunit, α L1 (the subunit is called ARL2) and a β -like subunit, ARL1 (Marshall *et al.*, 1990). A further four α -like subunits and two β -like subunits have been isolated from *Locusta* (Hermsen *et al.*, 1991) but as yet their sequences have not been published. Insect subunits whose cDNA sequences have been published are summarised in Table 1.4.

Hereinafter, the second α -like subunit from *Drosophila* is called D α 2/SAD (except in alignments where it is termed SAD) and the α -like subunit from *Schistocerca* is called α L1 (except in alignments where it is termed ARL2).

Generally, the mRNAs encoding the subunits displayed in Table 1.3 are approximately 2-3kb. After cleavage from the putative signal peptide, the mature proteins comprise 493 (SBD), 497 (ARD), 534 (α L1), 535 (D α 2/SAD) and 546 (ALS) amino acids.

| Insect | α -like | β -like |
|---------------------|-------------------------|----------------------|
| <i>Drosophila</i> | ALS D α 2/SAD | ARD (AChR64B) SBD |
| <i>Schistocerca</i> | α L1 | |

Table 1.4. Published insect cDNA sequences encoding nAChR subunits. ALS: Bossy *et al.*, 1988; D α 2: Baumann *et al.*, 1990, Sawruk *et al.*, 1990a; ARD: Hermans-Borgmeyer *et al.*, 1986, Wadsworth *et al.*, 1988; SBD: Sawruk *et al.*, 1990b; α L1: Marshall *et al.*, 1990.

The subunits contain between one and three potential N-glycosylation sites. Of these, one at amino acid position 24 is conserved in all insect subunits. To date, this site is characteristic of all neuronal nAChRs and is missing from all muscle nAChRs and the α 9 subunit. Putative protein phosphorylation sites have been predicted in the cytoplasmic loop for all the *Drosophila* subunits. Sites for protein kinase A have been predicted for ALS and ARD; for protein kinase C have been predicted for ALS and D α 2/SAD and for tyrosine protein kinase have been predicted for SBD, suggesting that phosphorylation of subunits may play a role in the regulation of insect nAChRs similar to that seen in vertebrates (reviewed in Gundelfinger and Hess, 1992). In *Schistocerca*, protein kinase C sites have been predicted in the cytoplasmic loop.

The insect subunits displayed in Table 1.4 share relatively high sequence identity with each other, as shown in Table 1.5. D α 2/SAD and α L1 show the greatest overall identity, suggesting that they are orthologs, i.e. they have arisen from the same gene in different species (le Novère and Changeux, 1995). It can be seen that α -like subunits do not always have higher homology with other α -like subunits than β -like subunits and vice versa. For example, SBD has greater sequence identity with both *Drosophila* α -like subunits than ARD which has roughly equal identity with all subunits. Closer examination of the alignment shown in Figure 1.6 indicates that the sequence similarities are unevenly distributed along the protein. Highest similarity is seen in the extracellular region and the transmembrane domains whereas the cytoplasmic loop between TM3 and TM4 and the C-terminal tail show significantly lower sequence similarities between subunits. Interestingly, the sequence similarity of insect subunits is higher with vertebrate neuronal receptors than with those subunits expressed in vertebrate muscle (see alignment in Marshall *et al.*, 1990).

1 50
Drosophila SAD MAPG**CCT**TRPRPIALLAHIW**RHCKPL**CLLLVLLLL**CETVQANP**DAKRLY**DDLLSNYNRLIRPVSNN**TD**TVLVKLGRLSQLIDLNLKDQILTTNVWLEHE**
Schistocerca ARL2MAAAPL...PMLLLLLLLLLLHHPAAAN**PDAKRLYDDLLSNYNRLIRPVSNN**TD**TVLVKLGRLSQLIDLNLKDQILTTNVWLEHE**
Drosophila SBDAPIS**FEANPDT**KRLY**DDLLSNYNRLIRPVVNN**TETL**TVWLGLKLSQLIEVNLKNQVM**TTNLWVKQR
Drosophila ALSMGSVLFAAVFIALHFATGGL**.ANPDAKRLYDDLLSNYNRLIRPVGNNSDRLTVKMGLRSQLIDVNLKNQIM**TTNVWVEQE
Drosophila ARDME**SSCK**SWLL**CS**ILVLVAFSLV**SASEDEER**LVRDLFRGY**NKLIRPVQNM**TQKVGVRFG**LAFLVQLIN**VNEKNQVMK**SNVWLRLV**

100 150
Drosophila SAD WQDHKFKWD**PSEYGGVTELYVPSEHIWLPDIVLYNNADGEYVV**TTMTKAILHY**TGKVVWTPPAIFKSSCE**IDVRYFPFDQQT**CFMKFGSWTYDGDQIDLK**
Schistocerca ARL2 WQDHKFRWD**PAEYGGVTELYVPSEHIWLPDIVLYNNADGEYVV**TTMTKAVLHHT**GKVVWTPPAIFKSSCE**IDVRYFPFDQQT**CFMKFGSWTYDGDQIDLK**
Drosophila SBD WFDYKLRWD**PEEYGGVEQLYVPSEHIWVPDIVLYNNWDGNYEVT**LMTKATLK**YTGEVFWEPPAIYKSSCE**MNV**EYFPYDEQICFMKFGSWTYNGA**QVDLK
Drosophila ALS WNDYKLRWD**PDDYGGVDTLHVPSEHIWHPDIVLYNNADGNYEVT**IMTKAILHHT**GKVVWKPPAIYKSFCE**IDVEYFPFDQQT**CFMKFGSWTYDGYMVDLR**
Drosophila ARD WYDYQLQW**DEADYGGIGVLR**LPPDKVWKP**DIVLFNNADGNYEVRYKSNVLIYPTGEVLWVPPAIYQSSCT**IDVTYFPFDQQT**CFMKFGSWTFNGDQVSLA**

200 250
Drosophila SAD HISQKNDKDNKVEIGID**LDREYYPSVEWDILGVPAERHEKYYPCCAEPYP**DIFFNITLRRKTLFY**TVNLIIPCVGISYLSVLVFYLPADS**G**GEKIALCISIL**
Schistocerca ARL2 HINQKYD.DNKVKVGID**LDREYYPSVEWDILGVPAERHEKYYPCCAEPYP**DIFFNITLRRKTLFY**TVNLIIPCVGISYLSVLVFYLPADS**G**GEKIALCISIL**
Drosophila SBD HLDQIPGSNL.VQVGID**LDTEFYLSVEWDILEVPATKNEEYYPDTLEPFS**DI**TFKLTMRKTLFYTVNLIIPCV**AL**TF**TLVLVFYLP**SDS**G**GEKVTL**CISIL
Drosophila ALS HLKQTADSDN.IEVGID**LDQYYISVEWDIMRVP**AVRNEKFY**SCCEEPYL**DIVFNLTLRRKTLFY**TVNLIIPCVGISF**LSVLVFYLP**SDS**G**GEKISL**CISIL
Drosophila ARD LYNNKN.....FVDLS**DYWKS**GTWDII**EV**PAYLNVYEGDSN**HP**TETDITFYIIIRKTLFY**TVNLILPTVLI**SFL**CV**LVFYLP**AEAGEKVTL**GISIL

300 350
Drosophila SAD LSQTMFFLLISEIIP**STSLALPLL**GKYL**FTMLLVGLSVVITIIILNIHYRK**PTHKMRPWIR**SFFIKRLPKLLM.RVPKDLLRDLAANKI...NYGLK**
Schistocerca ARL2 LSQTMFFLLISEIIP**STSLALPLL**GKYL**FTMVLVGLSVVITIMVLNVHYRK**PTHKMAPWVRKV**FIRRLPKLLM.RVPEQLLADLASKRLLRHAHNSK**
Drosophila SBD VSLTVFFLL**LA**EIIP**PTSLAVPLL**GKYL**FTMILVLSVWTTVCVLNIHFRSP**THNMSRLVRKL**FLHFMPKLM**MMRRTQ.....YTLP
Drosophila ALS LSLTVFFLL**LA**EIIP**PTSLTVPLL**GKYL**FTMMLVTL**SVV**TVIAVLNVNFRSPV**THRMAPWV**QRLFIQILPKLLC**IERPK**KEEPEEDQ**PEVLT**DVYHLP**
Drosophila ARD LSLVVFLLLVSKIL**PPTSLV**LPLIAKYL**FTFIMNTVSILVTVIIINWNFRGPR**THRMPMYIRSIFLHYLPAFL**FMKRPRKTRLRWMMEMP**GMSMPAPH

| | | |
|--------------------------|---|-----|
| | 400 | 450 |
| <i>Drosophila</i> SAD | FSKTKFGQALMDEMOMNSGGSSPDSLRR.....MQGRVGAGGCNGMHVTTATNRFSGLVGALGGGLSTLSGYNGLPVLSGLDDSLSDVAAR | |
| <i>Schistocerca</i> ARL2 | LSAA..AAAAVAAAASSSAASSPDSLRRHHHLHQHQHHLQLHHLQRPGGCNGLH..SATNRFSGGSAGAFG.....GLPSVV.GLDGSLSDVATR | |
| <i>Drosophila</i> SBD |DYDDSTPSN.....GYTNEIDVRDSISD.....FPSE..FKDSQDGAYDNGMQNSVDSDNVIP..... | |
| <i>Drosophila</i> ALS | PDVDKFVNYDSKRFSGDYGI PALPA.....SHRFDLAAAGGISAHCFAPPLPSSLPLPGADDDLFSPSGLNGDISPGCCPAAAAAAAADLSPTFE | |
| <i>Drosophila</i> ARD | PSYGSPAELPKHISAIGGKQSKMEVMEISDLH.....HPNCKINRKVNSSGGEELGLGDGCRRESESSDSIL..... | |
| | | |
| | 500 | 550 |
| <i>Drosophila</i> SAD | KKYPFELEKAIHNVMFIOHHMQRQDEFNAEDQDWGFVAMVMDRLFLWLFMIA SLVGT FVILGEAPSLY...DDTKAIDVQLSDVAKQIYNLTEKKN..... | |
| <i>Schistocerca</i> ARL2 | KKYPFELEKAIHNVLFIONHMQRQDEFDAEDQDWGFVAMVLDRLFLWIFTIASIVGTFAILCEAPALY...DDTKPIDMELSSVAQQQF.LPDIDF..... | |
| <i>Drosophila</i> SBD | RNLTPVQLALRAVRFIAQH IKDADKDNEIVEDWKVFSMVLDRFFLWLF T LSCVFGTLAIICQSPSLY...DTRSPIDRQLSEIPLRKNNFM.LPPDIVRQVLT | |
| <i>Drosophila</i> ALS | KPYAREMEKTI EGSRFIAQHVKNKDKFESVEEDWKYVAMVLD RMFLWIFAIACVVG TALII LQAPSLH...DQSQPIDILYSKI AKKKFELLKMGSENTL.... | |
| <i>Drosophila</i> ARD | ..LSP EASKATEAVEFIAEHLRNEDLYIQ TREDWKYVAMVIDRLQLYIFFIVTTAGTVGILMDAPHIFEYVDQDRIIEIYRGK..... | |

Figure 1.6. Alignment of insect α -like and β -like subunits of the nAChR. Alignment constructed using the gcg program Pileup (Feng and Doolittle, 1987; Higgins and Sharp, 1989) after extracting amino acid sequences from the Genbank and EMBL databases.

| | D α 2/SAD | α L1 | SBD | ALS | ARD |
|------------------|------------------|-------------|-----|-----|-----|
| D α 2/SAD | 100 | 82 | 56 | 55 | 43 |
| α L1 | | 100 | 57 | 56 | 43 |
| SBD | | | 100 | 59 | 45 |
| ALS | | | | 100 | 42 |
| ARD | | | | | 100 |

Table 1.5. Amino acid identities between cDNA encoding insect α -like and β -like subunits. Identity calculated using the gcg program Distances (uncorrected) (Swofford and Olsen, 1990) from an alignment of these subunits. SwissProt accession numbers: D α 2/SAD p17644; α L1 p23414; SBD p25162; ALS p09478; ARD p04755.

Of the genes encoding insect subunits, D α 2/SAD is 5.5kb (Jonas *et al.*, 1990), ARD is over 7kb (Sawruk *et al.*, 1988) whilst ALS exceeds 54kb (Bossy *et al.*, 1988). Comparison with the known structures of vertebrate neuronal and muscle genes emphasises the closer relationship between insect subunits and vertebrate neuronal subunits than vertebrate muscle subunits. After exclusion of genes encoding the vertebrate neuronal α 7 (Couturier *et al.*, 1990b) and α 9 subunits (Elgoyhen *et al.*, 1994) which differ markedly from the other neuronal subunits and the muscle subunits, the remaining vertebrate neuronal genes α 2 - α 5 have the same exon/intron organisation (Nef *et al.*, 1988; Boulter *et al.*, 1990; Couturier *et al.*, 1990a), containing five introns within the protein coding region. Vertebrate muscle genes have all these introns and between three and six additional ones (Noda *et al.*, 1983a,b; Nef *et al.*, 1984; Buonanno *et al.*, 1989). The *Drosophila* genes have three (D α 2/SAD) or four (ALS and ARD) of these common introns, in addition to one (ARD) or three (D α 2/SAD and ALS) others, all of which differ from those in genes expressed in vertebrate muscle (reviewed in Gundelfinger, 1992; Gundelfinger and Hess, 1992). Comparison of the *Drosophila* α -like subunits with the neuronal α 2 - α 5 subunits shows that for these genes, the sequences corresponding to the first three transmembrane domains are part of the same exon, in contrast to those genes encoding the muscle subunits which are split into two or three exons. The intron/exon boundaries are summarised in Figure 1.7. Interestingly, the genes encoding ALS, D α 2/SAD and SBD have been mapped by *in situ* hybridisation to the same chromosomal band in the 96A region of the 3R *Drosophila* chromosome, suggesting that these genes have arisen by gene duplication and might form a gene cluster (Bossy *et al.*, 1988; Jonas *et al.*, 1990; Sawruk *et al.*, 1990b). In comparison,

ARD has been located in the 64B region of the 3L *Drosophila* chromosome (Sawruk *et al.*, 1988; Wadsworth *et al.*, 1988). The cluster of genes encoding ALS, D α 2/SAD and SBD is similar to that seen for the rat and chick α 3, α 5 and β 4 encoding genes (Boulter *et al.*, 1990; Couturier *et al.*, 1990a), which also form a gene cluster spanning 60kb in rat and 28kb in chick.

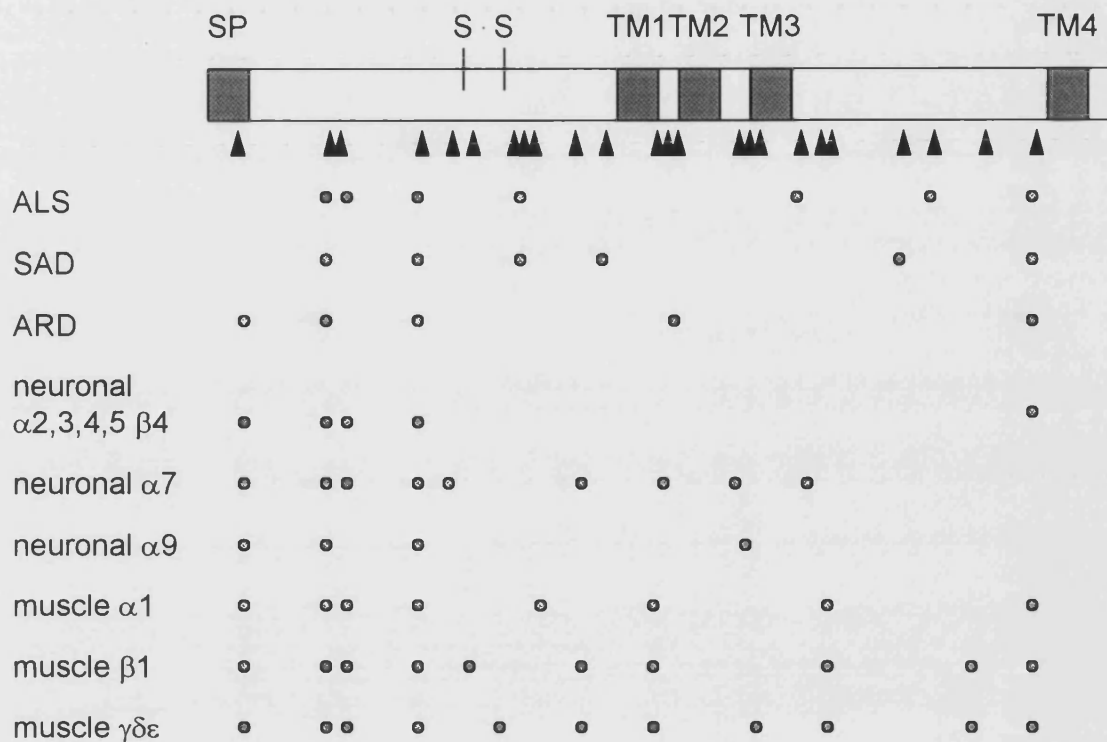


Figure 1.7. Comparison of intron positions for genes encoding *Drosophila* α -like and β -like subunits and vertebrate neuronal and muscle subunits. Arrowheads indicate intron positions on diagram of generic subunit (not to scale). Introns are indicated by circles. Abbreviations: SP, signal peptide; TM1-4, transmembrane regions 1-4, S-S, disulphide bridge representing cyst loop. Adapted from Gundelfinger, 1992 using data from Nef *et al.*, 1984; Bossy *et al.*, 1988; Nef *et al.*, 1988; Sawruk *et al.*, 1988; Buonanno *et al.*, 1989; Couturier *et al.*, 1990a,b; Jonas *et al.*, 1990; Elgoyhen *et al.*, 1994.

Fragments of genes encoding nAChR subunits have been isolated from a variety of insects, using PCR with degenerate primers to the extracellular region (shortly before the vicinal cysteines) and the beginning of TM3, based on the gene sequences encoding ALS, D α 2/SAD and α L1 subunits (Sgard *et al.*, 1993). At the amino acid sequence level, these fragments were clearly ALS-like or D α 2/SAD-like, indicating that other insect species possessed genes encoding nAChR subunits similar to those of *Drosophila*. Of the insects tested, only the peach potato aphid, *Myzus persicae* appeared to have gene

fragments encoding both subunits. Gene fragments isolated from the lepidopteran species used (*Chilo suppressalis*: the asiatic rice borer, *Heliothis virescens*: the tobacco budworm and *Spodoptera frugiperda*: the fall armyworm) were all closer to the ALS sequence than the Dα2/SAD sequence. The greatest homology was seen between *Heliothis virescens* and *Spodoptera frugiperda*, the two most closely related species. Both these insects possessed an intron within this gene fragment that was absent in *Chilo suppressalis*. *Manduca* was not used in this study.

1.3.3 Functional analysis of cloned genes and cDNAs

Although the homology between the cloned genes and cDNAs in insects and vertebrates suggests that they form nAChR subunits, only their assembly into receptors identified at the physiological and pharmacological level (see section 1.3.1) indicates their ability to form functional nAChRs. For insect nAChRs this has taken the form of immunoprecipitation of [¹²⁵I]α-btx binding sites and expression in *Xenopus* oocytes.

Immunoprecipitation of [¹²⁵I]α-Bungarotoxin binding sites

Bacterial fusion proteins have been used as antigens for the production of antisera against the *Drosophila* subunits, ALS and ARD. Antibodies against the cytoplasmic loop, which appears to be the most variable region of the nAChR subunits and hence specific for each subunit have been extremely useful in correlating cloned genes with [¹²⁵I]α-btx binding sites representing physiological nAChRs. Antisera against bacterially expressed fusion proteins that included the extracellular region (amino acids 65-212) and the cytoplasmic loop (amino acids 305-444) of ARD were used in immunoprecipitation experiments against [¹²⁵I]α-btx binding sites (Schloss *et al.*, 1988). The antisera precipitated between 20 and 30% of all the [¹²⁵I]α-btx binding sites in the *Drosophila* head solubilised membranes, suggesting that ARD antisera may bind exclusively to high affinity class 1 [¹²⁵I]α-btx binding sites which represent ~25% of the total toxin binding components. This was confirmed by Scatchard analysis which revealed that only class 1 binding sites (rather than class 2; see section 1.3.1) were removed specifically by ARD antibodies. Accounting for the facts that the ARD sequence did not contain the two vicinal cysteines nor did the ARD antisera actually interfere with toxin binding to *Drosophila* membranes, it was suggested that ARD was a structural subunit of the class 1 α-btx binding nAChR in *Drosophila*.

Bacterial fusion proteins containing the ALS extracellular regions corresponding to amino acids 192-212 and 135-224 were shown to bind [¹²⁵I]α-btx but with a lower affinity than

that of native class 1 binding sites (Ohana and Gershoni, 1990; Schloss *et al.*, 1991). Antisera against a fusion protein of the cytoplasmic loop of ALS (amino acids 327-463) immunoprecipitated between 20 and 30% of the [125 I] α -btx binding sites from *Drosophila* head solubilised membranes, suggesting that ALS was associated with the class 1 high affinity [125 I] α -btx binding sites, also recognised by ARD-specific antisera (Schloss *et al.*, 1991). Combining equal amounts of ALS and ARD specific antisera again precipitated 20-30% of the [125 I] α -btx binding sites, confirming that ALS and ARD antisera recognise the same high affinity [125 I] α -btx binding site. These results suggested that ALS and ARD were subunits of the same heteromeric nAChR with ALS as a ligand binding subunit and ARD as a structural subunit.

Although these immunoprecipitation experiments have established the existence of ALS and ARD in the same receptor complex, they do not preclude the possibility of further subunits in the receptor. More comprehensive studies encompassing the range of *Drosophila* subunits (see section 1.3.2) are needed to account for the remaining α -btx binding sites. Given that this is a difficult technique to interpret, additional information may not make the situation any clearer.

Expression in Xenopus oocytes

Sawruk *et al.* (1990a) demonstrated that D α 2/SAD cRNA, expressed in *Xenopus* oocytes formed cation channels that were gated by nicotine and thus was able to form an homomeric receptor. However, the nicotine concentrations required to elicit responses were high (half maximal responses were seen at 10-15mM agonist rather than micromolar concentrations observed for example in the homomeric chick and rat α 7 receptors; Couturier *et al.*, 1990b, Seguela *et al.*, 1993) and both 100 μ M d-tubocurarine and 1 μ M α -btx failed to block the responses, suggesting the formation of a rather unphysiological receptor that might require other subunits for correct formation and physiological function. Co-expression of D α 2/SAD with ARD did not alter these unusual properties indicating either that ARD was not the appropriate subunit or that other subunits in addition to ARD were necessary.

Further evidence to support the existence of functional homomeric receptors that operated under physiological conditions was gained with the expression of α L1 cRNA and cDNA (Marshall *et al.*, 1990; Amar *et al.*, 1995). However, the data from these two studies cannot be easily compared as in Marshall's report, the responses measured were membrane potentials whereas in Amar's report the responses measured were membrane currents under voltage clamp. This may explain the differences seen in response to

nicotine when α L1 alone was expressed in *Xenopus* oocytes. In each case, functional ion channels were formed that were gated by nicotine, but at micromolar concentrations according to Marshall *et al.* and at millimolar concentrations (EC_{50} : 0.83mM) according to Amar *et al.* Nicotine evoked currents were blocked by the nicotinic antagonists α -btx and n-btx (each at 0.1 μ M), in addition to a more unusual blockade by bicuculline and strychnine (Marshall *et al.*, 1990) and by MLA (0.1 μ M; Amar *et al.*, 1995). These pharmacological characteristics were all consistent with data from insect neuronal recordings *in situ* (Sattelle *et al.*, 1983; Benson, 1988; Pinnock *et al.*, 1988; Chiappinelli *et al.*, 1989 and Buckingham *et al.*, 1994). It was therefore concluded that the α L1 cDNA encoded a subunit of a locust α -btx sensitive functional receptor. It is notable that the α -btx core binding site (see section 1.2.7) is perfectly conserved between α L1 and D α 2/SAD.

Preliminary experiments on the expression of ALS and ARD in *Xenopus* oocytes demonstrated that alone, neither could generate nicotine-evoked responses but that together a functional heteromeric receptor was formed (discussed in Schloss *et al.*, 1991). More recently, Bertrand *et al.* (1994) have reported that ALS alone and in combination with ARD failed to reconstitute functional nAChRs when expressed in *Xenopus* oocytes. In contrast, co-expression of ALS with the chick β 2 subunit did produce functional nAChRs. The resulting ACh evoked currents were large, with a maximum amplitude of 2 μ A whereas at the same concentration, nicotine evoked currents were ~20% smaller. Dose response curves gave an EC_{50} of 0.18 μ M for ACh and 1.3 μ M for nicotine. Furthermore, ACh evoked currents were blocked by 100nM α -btx. The same workers also found that D α 2/SAD did not form functional nAChRs in *Xenopus* oocytes either alone or in combination with ARD or with ALS and ARD but when co-expressed with the chick β 2 subunit reconstituted functional nAChRs. Similarly to ALS/ β 2, ACh evoked responses were larger than nicotine evoked responses, however lower affinities were demonstrated with an EC_{50} of 35 μ M for ACh and 7.4 μ M for nicotine. In contrast to ALS/ β 2, ACh evoked responses were not blocked by 100nM α -btx and desensitised faster. These results indicated that ligand binding and desensitisation properties were due to the α -like subunit, as the same β subunit was used in both cases. As functional expression was only demonstrated with inclusion of the β subunit, it was also suggested that ALS and D α 2/SAD formed heteromeric receptors rather than the homomeric receptor previously described for D α 2/SAD (Sawruk *et al.*, 1990). This discrepancy was attributed to the use of physiological concentrations of ACh in this study rather than the high concentrations used previously. Finally, the fact that co-expression of each α -like subunit with ARD failed to reconstitute functional nAChRs but their

association was known *in vivo* (Schloss *et al.*, 1991; Schuster *et al.*, 1993) and that the ARD subunit was significantly expressed in the oocytes as demonstrated by immunocytochemistry localisation (Bertrand *et al.*, 1994) implied that additional structural subunits were needed for a functional receptor.

It is also possible that the α L1 subunit is heteromeric in the native receptor. Although it is capable of forming functional homomeric ion channels, it may need additional structural subunits for responses to be evoked at physiological concentrations (Amar *et al.*, 1995). A similar suggestion was made for the chick α 7 receptor (Couturier *et al.*, 1990b; see section 1.2.5, Functional expression of neuronal nAChR genes). Given that the same subunit can form either homomeric or heteromeric receptors in different hands, it is important to point out that expression in *Xenopus* oocytes is not a straightforward technique and its success depends on many factors. These are discussed in Chapter 5.

For simplicity, the results of expression are summarised in Table 1.6.

| | Currents evoked by ACh ? | Currents evoked by nicotine ? | Currents blocked by α -btx ? |
|-----------------------------|--------------------------|-------------------------------|-------------------------------------|
| ALS [•] | NO | NO | NO |
| SAD [■] | YES | YES | NO |
| α L1 [♦] | YES | YES | YES |
| ARD | ND | ND | ND |
| ALS/ARD [•] | NO | NO | NO |
| SAD/ARD ^{■♦} | NO | NO | NO |
| ALS/SAD/ARD [•] | NO | NO | NO |
| ALS/ β 2 [•] | YES | YES | YES |
| SAD/ β 2 [•] | YES | YES | NO |

Table 1.6. Expression of insect subunits in *Xenopus* oocytes. ND, not determined. [•]Bertrand *et al.*, 1994; [■]Sawruk *et al.*, 1990a; [♦]Marshall *et al.*, 1990, Amar *et al.*, 1995.

Localisation by immunohistochemical studies

The distribution of ALS and ARD in *Drosophila* has been studied by Schuster *et al.* (1993). Using subunit specific antibodies targeted to the cytoplasmic loop between TM3 and TM4, co-localisation in many areas of the neuropile (with the exception of only ARD immunoreactivity in the lamina of the optic lobe) was demonstrated. During embryonic

development, ARD immunoreactivity was first detected in ~10 hour embryos whereas both subunits were detected in the CNS of late embryos, the three larval stages and all prepupal and pupal stages. In larvae and adults, immunoreactivity to both ALS and ARD was heavily concentrated in the synaptic regions while embryos and pupae also exhibited strong immunoreactivity in the cortical cell bodies. It was suggested that, as these developmental periods coincided with accumulation of ARD transcripts, cell body staining could reflect newly synthesised and assembled receptors whilst functional, heteromeric ALS and ARD containing receptors could be intended for synapses.

In contrast to adult *Drosophila*, a polyclonal antibody raised against the purified, homomeric α -btx binding protein from *Locusta* (Breer et al., 1985) demonstrated immunoreactivity in both synaptic and cell body membranes of the neuropile in the CNS of the adult *Schistocerca* (Leitch et al., 1993), as well as in particular glial cells. Analysis of the distribution of immunoreactivity to the same antibody in developing *Schistocerca* showed that labelling first appeared between 30 to 35% development on axonal outgrowths from differentiated neurons which at 40 to 45% development appeared in the cytoplasm of neuronal cell bodies. By 50% development when the developing neuropile was enclosed this labelling was uniform but soon after areas representing the location of future tracts and commissures were unlabelled. The adult pattern of immunoreactivity was first observed at 75% development when synaptic neuropile regions were stained but tracts and commissures were not, suggesting that at early stages of development nAChR-like antigenic sites were evenly distributed but were later concentrated to the synaptic regions (Watkins et al., 1995).

Temporal and spatial localisation

Genes and cDNAs that encode functional nAChRs would be expressed specifically in the nervous system and this has been demonstrated for several nAChR genes from *Drosophila*. During development, the expression of putative nAChR genes appears to be highly regulated. Transcripts from genes encoding ALS, D α 2/SAD, ARD and SBD have been detected on Northern blots of developmental stages of *Drosophila*, beginning with mid-late aged embryos to adult flies. Levels of ARD, D α 2/SAD and SBD were particularly high in late embryogenesis and in late pupae but had decreased by the early adult stage and were only detected at very low levels in larvae and late adults (Hermans-Borgmeyer et al., 1986; Wadsworth et al., 1988; Sawruk et al., 1990a,b). In contrast, the level of ALS transcript remained at elevated levels in larvae (Bossy et al., 1988). The periods of high expression of the ARD transcripts corresponded to periods of terminal neuronal differentiation and synapse formation in the CNS, suggesting that they were of neuronal origin (Hermans-Borgmeyer et al., 1986). This was also implied from *in situ*

hybridisation which showed that transcripts from the ARD, D α 2/SAD and SBD *Drosophila* putative nAChR genes were expressed throughout the CNS but not in non-CNS regions (Wadsworth *et al.*, 1988; Sawruk *et al.*, 1990a,b). Comprehensive studies for ARD transcripts showed that transcripts were first detected in neuronal cell bodies of 10-12 hour embryos corresponding to condensation of the ventral nerve cord. Transcript levels increased during late embryogenesis in all parts of the CNS and then decreased in larvae before rising again in both the cortical regions of the brain and in the thoracic ganglion of the pupae. In adult flies, ARD transcripts were expressed in all cortical regions of the neuropile except the lamina of the optic lobes (Wadsworth *et al.*, 1988).

Of other insects investigated, Hermesen *et al.* (1991) have shown that transcription of *Locusta* nAChR subunits (sequences unpublished) begins during late embryonic development.

1.4 Summary

The wealth of evidence described here strongly suggests the presence of excitatory nicotinic cholinergic receptors within the insect CNS. Moreover, it is clear that an heterogeneity of subtypes exists, apparent at the pharmacological, physiological and molecular level. However, this does not preclude the existence of an homomeric receptor, postulated to be the structure of an ancestral ligand gated ion channel (Lunt, 1986).

There is abundant evidence to support cholinergic transmission in *Manduca*. The possibility of nicotine resistance in *Manduca* as a result of a 'mutated' nAChR will only be solved through pharmacological characterisation of binding sites on brain membranes and isolation of genes and/or cDNAs encoding putative nicotine resistant nAChRs and their subsequent expression in a functional system. Consequently, the aims of this study were to isolate a gene(s) or cDNA(s) encoding a putative α -like nAChR subunit(s) (i.e. those subunits generally thought to bind nicotine in vertebrates and other invertebrates) and correlate the sequence(s) obtained with pharmacological profiles from radioligand binding studies on *Manduca* adults and larvae. The results obtained may help to establish the true cause of *Manducas* nicotine insensitivity and may give further insight into the nicotine binding site of the nAChR.

CHAPTER 2

Molecular characterisation of an α -like subunit of the nAChR from *Manduca sexta*

2.1 Introduction

Morris (1984) and Trimmer and Weeks (1989) have postulated that the reduced nicotine sensitivity seen in *Manduca* larvae could reflect, in part, an altered nAChR, making it a natural, nicotine-insensitive, mutant receptor. A plausible explanation of the nicotine insensitivity is a mutation resulting in an alteration of a key amino acid in the binding site on the extracellular region of the α -like subunit (reviewed in Devillers-Thiéry *et al.*, 1993; further discussion in Chapter 1, sections 1.1.3 and 1.2.7) relative to the α -like subunits of other nicotine sensitive insects.

A partial length cDNA encoding an α -like subunit, named MARA1 (for *Manduca* Acetylcholine Receptor Alpha-like subunit 1) had been cloned previously from a cDNA library prepared from developing (9-12 day old) adult brains (Clarke, 1991) by cross-hybridisation with the α -like subunit of the nAChR from the locust, *Schistocerca gregaria*, α L1 (Marshall *et al.*, 1990).

The sequence, hereinafter called fragment c, contained the cys loop common to all subunits of the nAChR, the two vicinal cysteines indicative of an α -subunit, the four putative transmembrane domains (TM1-4), the intracellular loop between TM3 and TM4 and part of the N-terminal extracellular region. Fragment c lacked some 5' sequence of the coding region, equivalent to approximately 90 N-terminal amino acids (from comparison with other insect α -like subunits; see sequence alignment, Figure 2.36) and any 5' untranslated sequence. Fragment c is shown in Appendix 2, part a.

In order to test the hypothesis that the reduced nicotine sensitivity seen in *Manduca* is due to a naturally mutated nAChR, and assuming that MARA1 contains the necessary altered amino acid residue(s) of this mutated nAChR, it was necessary to isolate the missing N-terminal fragment of MARA1 and find or construct a full-length clone of MARA1 before investigating its nicotine sensitivity in an expression system.

This chapter therefore, delineates the steps taken to determine the full coding sequence and the attempts made to isolate a full-length clone of MARA1. Several techniques were used, including cDNA library screening and a variety of PCR based strategies and these are discussed briefly below.

cDNA libraries are widely used as molecular tools for finding particular cDNA clones. They are easier to manipulate than genomic libraries, as in theory, a cDNA clone is more

amenable to identification than the equivalent gene. As cDNA clones are representative of the mRNA in the original preparation, a cDNA library from adult *Manduca* brain should contain a proportion of clones representing nAChRs and ideally that of MARA1.

PCR based strategies offer an efficient alternative to time consuming library screens and are useful in cases where the gene or cDNA of interest is expressed at low levels or if a limited amount of material is available because the DNA is amplified through the PCR. The inverse PCR technique is beneficial in obtaining the 5' ends of cDNA or genes as it relies on the digestion and circularisation of the genetic material such that the primers used in amplification effectively synthesise nucleotides from the inside to the outside of the known sequence (Towner and Gärtner, 1992). A further alternative is to use RACE PCR (rapid amplification of cDNA ends; Frohman *et al.*, 1988) or modifications thereof. In this technique, either an homopolymeric tail or an oligonucleotide of known sequence is ligated to the cDNA. Primers designed to the 5' end of the homopolymeric tail or the oligonucleotide and the 3' end of the cDNA are then used to amplify the cDNA.

2.2 Materials

Molecular biology grade chemicals were obtained from Fisons Scientific Equipment, Loughborough, U.K. Bacteriological culture media and Tris-saturated phenol:chloroform:isoamyl alcohol were obtained from the Sigma-Aldrich Chemical Company Ltd., Poole, U.K. Water saturated phenol was from Rathburn Chemicals Ltd., Walkburn, U.K. Absolute ethanol was obtained from Hayman Ltd., Witham, U.K. Ultrapure Sequagel™ Sequencing Systems acrylamide solutions were obtained from National Diagnostics, Hull, U.K. Falcon plasticware was supplied by Becton Dickinson and Company, Plymouth, U.K. and Nunc plasticware by Life Technologies, Glasgow, U.K. Whatman 3MM Chromatography Paper was from Whatman International Ltd., Maidstone, U.K.

Restriction and modifying enzymes were purchased from New England Biolabs, Hitchin, U.K. or from the Promega Corporation, Southampton, U.K. with the exception of T4 DNA Ligase and SuperScript™ II RNase H- Reverse Transcriptase which were purchased from GibcoBRL, Paisley, U.K. and Expand™ Long Template PCR System and RNase Inhibitor from Boehringer Mannheim U.K., Lewes, U.K.

Ultrapure dNTP sets, ³²P-QuickPrime™ Kits and MicroSpin™ columns were obtained from Pharmacia Biotech, St. Albans, U.K. Wizard™ Minipreps DNA Purification System Kits were purchased from Promega and Sequenase™ Version 2.0 DNA Sequencing Kits (United States Biochemical) from Amersham International Plc., Little Chalfont, U.K. GeneClean® II Kits (Bio101 Inc.) and Kodak X-Omat LS™ film were from Anachem, Luton, U.K. High Pure PCR Product Purification Kits, positively charged nylon membranes and the non-radioactive DIG System Labelling and Detection Kits were purchased from Boehringer Mannheim. Dynabeads® mRNA Purification Kits were supplied by Dynal®, Wirral, U.K. The GeneAmp® Thermostable *rTth* Reverse Transcriptase RNA PCR Kit was purchased from Perkin Elmer Ltd., Warrington, U.K. Radioisotopes were from NEN-Dupont (U.K.) Ltd., Stevenage, U.K.

Oligonucleotides were synthesised either in house, by R&D Systems Europe Ltd., Abingdon, U.K. or by Perkin Elmer Ltd.

A customised cDNA library in the vector λ ZapII, was made by Clontech Laboratories Inc., (Cambridge Bioscience), Cambridge, U.K., using *Manduca* heads from the pupal stage, dissected by Professor S.E. Reynolds. A partial cDNA of an α -like subunit of the nAChR

from *Manduca* (MARA1) in the vector pBluescript was kindly donated by B. Clarke (Shell Research Ltd., Sittingbourne, U.K.). *Manduca* eggs were supplied by the University of Bath *Manduca* colony. Rat genomic DNA was donated by Dr. S. Wheeler (University of Bath, U.K.).

2.3 Methods

2.3.1 Techniques used in nucleic acid preparation and synthesis

Calcium chloride competent cells

A 5ml culture of either *E. coli* XL-1 blue cells or DH5 α cells in 2YT (see Appendix 3) was incubated with shaking at 37°C overnight. 200 μ l of overnight culture was added to 50ml 2YT and incubated with shaking at 37°C until an A₅₅₀ of 0.3 was reached. The cells were placed on ice for 5min, centrifuged in a Sorvall SS34 rotor at 1090g, 4°C for 5min and the pellet resuspended in 20ml 100mM CaCl₂. After a further 20min on ice, the cells were centrifuged as above and the pellet resuspended in 2ml 100mM CaCl₂.

Transformation

DNA (50-100ng) was added to 200 μ l competent cells and incubated on ice for 40min. The cells were heat shocked at 42°C for 90s, 1ml 2YT was added and the cells incubated at 37°C for 40min before being plated onto 2YT agar plates containing ampicillin at 100ng/ml for transformant selection. Plates were then incubated at 37°C overnight.

α -Complementation

Transformed competent cells which utilised the replacement of the lacZ gene with foreign DNA were selected for on 2YT ampicillin plates supplemented with the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) and the inducer isopropylthio- β -D-galactoside (IPTG) as described in Sambrook *et al.* (1989).

Storage of bacterial cultures

Bacterial cultures were stored indefinitely at -20°C with the addition of 15% (v/v) sterile glycerol, as described in Sambrook *et al.* (1989).

Plasmid DNA preparation

'Mini' scale

5ml of 2YT media containing ampicillin at 100ng/ml was inoculated with either a single colony of transformed cells or 5 μ l of a glycerol stock and incubated with shaking at 37°C overnight. Plasmid DNA was subsequently prepared using the Promega Wizard™ Minipreps DNA Purification System as described in the manufacturer's protocol.

'Midi' scale

50ml of 2YT media containing ampicillin at 100ng/ml was inoculated with 200µl of glycerol stock and incubated with shaking at 37°C overnight. Cells were harvested by centrifugation in a Sorvall SS34 rotor at 7720g for 5min. The pellet was resuspended in 4ml of pre-lysis buffer (50mM glucose, 10mM EDTA, 25mM Tris-Cl, pH8.0) and the cells lysed with 4ml of alkaline lysis solution (0.2M NaOH, 1% (w/v) SDS). The alkali was neutralised by the addition of 4ml of neutralising solution (3M sodium acetate, pH4.8) and the mixture centrifuged in a Sorvall SS34 rotor at 17400g for 5min.

The supernatant was mixed with an equal volume of isopropanol and centrifuged at 17400g for 5min. The pellet was resuspended in 500µl of sterile water in a microcentrifuge tube and the alkaline lysis and neutralisation steps repeated using 250µl volumes in order to eliminate any remaining cellular DNA. The pellet obtained after a second isopropanol precipitation was resuspended in 500µl of sterile water and incubated for 5min with an equal volume of 6M LiCl in order to precipitate RNA. After centrifuging at high speed for 2min the DNA in the supernatant was ethanol precipitated and the subsequent pellet resuspended in 400µl of TE, pH7.8 (see Appendix 3). RNA was digested by the addition of DNase-free RNase to a concentration of 100µg/ml and incubation at 37°C for 30min. The reaction was stopped by the addition of 20µl 10% (w/v) SDS and incubation at 75°C for 10min. The remaining RNA was then precipitated by incubation at room temperature with 6M LiCl for 15min and pelleted by centrifugation at high speed. DNA in the supernatant was ethanol precipitated and the subsequent pellet resuspended in sterile water before extraction of protein once with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). After a final ethanol precipitation, the DNA pellet was resuspended in 100µl sterile water.

Genomic DNA preparation

Genomic DNA was prepared according to the method of Herrmann and Frischauf (1987). *Manduca* eggs were ground to a powder in liquid nitrogen and dispersed in 9 ml TEN9 buffer (50mM Tris-Cl, pH9.0, 100mM EDTA, 200mM NaCl) containing 100µg/ml DNase-free RNase A. The solution was rocked at room temperature for 10min, 0.5ml 20% (w/v) SDS was added and rocked for a further 10min, followed by the addition of 0.5ml proteinase K (10mg/ml in sterile water). The solution was incubated at 55°C with shaking overnight. Equilibrated phenol (pH8.0; 10ml) was added and the solution rocked at room temperature for 2.5h. After centrifugation in a Sorvall SS34 rotor at 1090g, 25°C for 10min, the aqueous layer was removed and respun in a Sorvall SS34 rotor at 9770g,

25°C for 30min. The supernatant was dialysed against 1l of TE, pH7.8 at room temperature for 4h, then against 2l TE, pH7.8 at 4°C overnight. The DNA was precipitated with 0.1 volumes of 3M sodium acetate, pH5.5 and 0.8 volumes isopropanol. The solution was mixed gently and the DNA spooled out on a glass rod, washed with 70% (v/v) ethanol and dissolved in 0.5ml TE at 4°C over a week on a rotating wheel.

Total RNA isolation

Total RNA was isolated from day 2-3 *Manduca* eggs using the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Eggs (1g) were frozen and ground to a powder in liquid nitrogen, dispersed in 10ml denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH7.0, 0.5% (v/v) sarkosyl, 0.1M 2-mercaptoethanol), homogenised and transferred to a 30ml Corex tube. In succession, 1ml 2M sodium acetate pH4.0, 10ml water saturated phenol and 2ml chloroform:isoamyl alcohol (49:1) were added to the homogenate, mixed thoroughly and the suspension cooled on ice for 15min. After centrifugation in a Sorvall SS34 rotor at 12100g at 4°C for 20min, the aqueous phase was removed, mixed with 10ml isopropanol and precipitated at -20°C for 2h. The RNA was centrifuged as above and the pellet resuspended in 3ml denaturing solution. The RNA was re-precipitated with 3ml isopropanol at -20°C overnight. The RNA was centrifuged as above, resuspended in 10ml RNase free 75% (v/v) ethanol, sedimented and dried. The RNA was dissolved in 1ml RNase free 0.5% (w/v) SDS for 10min.

Poly [A+] RNA isolation

Poly [A+] RNA was selected using Dynabeads® oligo (dT)₂₅ (DynaI®). These are uniform, superparamagnetic polystyrene beads with a 2.8µm diameter which have chains of deoxythymidylate, 25 nucleotides long, covalently attached to the surface of the beads via a 5' linker group.

An aliquot of total RNA (75µg) was heated to 65°C for 2min. 200µl (1mg) of resuspended Dynabeads® oligo (dT)₂₅ was added to a microcentrifuge tube and separated from the supernatant using the Dynal MPC®-E-1 magnetic separator. The supernatant was removed and the beads washed with 200µl 2x binding buffer (20mM Tris-Cl pH7.5, 1M LiCl, 2mM EDTA). The washed beads were then resuspended in 100µl 2x binding buffer. The heated total RNA was added to the beads, mixed and left to hybridise at room temperature for 5min. The supernatant was removed using the magnet and the beads washed twice with 200µl washing buffer (10mM Tris-Cl pH7.5, 150mM LiCl, 1mM EDTA). The beads were resuspended in 16µl elution buffer (2mM

EDTA, pH7.5), heated to 65°C for 2min and placed on the magnet. The eluted RNA was transferred to a new microcentrifuge tube and used directly for cDNA synthesis.

First strand cDNA synthesis

To 16µl poly [A+] RNA was added 1µl oligo dT linker primer (RoRi(dT)₁₇; sequence 5' GACTACGTTAGCATCTAGAATTCTCGAGT₁₇ 3'). This mixture was heated to 65°C for 2-10min and then chilled on ice before the addition of 6µl 5x 1st strand buffer (250mM Tris-Cl, pH8.3, 375mM KCl, 15mM MgCl₂), 3µl 0.1M DTT and 2µl 10mM dNTPs. After equilibration at 37°C for 2min, 1µl RNase inhibitor (40Units) and 1µl SuperScript™ II RNase H- Reverse Transcriptase (200Units) was added, mixed gently and incubated at 37°C for 1h.

In order to assess the size of the first strand cDNA, 1µl [α -³²P]dCTP was added to 0.1 volumes of the total reaction volume and incubated at 37°C for 1h.

Second strand cDNA synthesis and circularisation

40µl 5x second strand buffer (100mM Tris-Cl, pH7.5, 25mM MgCl₂, 50mM (NH₄)₂SO₄, 250µg/ml BSA), 3µl 10mM dNTPs, 131.2µl sterile water, 0.8µl RNase H (2Units/µl) and 5µl DNA Polymerase I (10Units/µl) was added to 20µl first strand cDNA. The contents of the tube were mixed and incubated at 16°C for 2.5h. cDNA was extracted once with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform, ethanol precipitated at -20°C overnight, sedimented at high speed for 1h, washed with 75% (v/v) ethanol, dried and resuspended in 38.5µl TE, pH8.0.

Double stranded cDNA was made blunt ended by the addition of 5µl T4 DNA Polymerase buffer (330mM Tris acetate, pH7.9, 660mM sodium acetate, 100mM magnesium acetate, 5mM DTT, 1mg/ml BSA), 3µl 2mM dNTPs and 3.5µl T4 DNA Polymerase (2.9Units/µl) and incubated at 37°C for 30min. Sterile water was added to give a total volume of 100µl and DNA extracted once with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform. DNA was ethanol precipitated at -20°C for 1h. After centrifugation as above, the pellet was washed with 75% (v/v) ethanol, dried and resuspended in 792µl sterile water.

The blunt ended cDNA was circularised with 200µl 5x T4 DNA Ligase buffer (250mM Tris-Cl, pH7.6, 50mM MgCl₂, 25% (w/v) PEG8000, 5mM ATP, 5mM DTT) and 8µl T4 DNA Ligase (5Units/µl) in a total volume of 1ml. The ligation was incubated at 16°C for

60h. The circularised DNA was ethanol precipitated at -20°C overnight and sedimented as above, washed, dried and resuspended in 50µl sterile water.

Single stranded cDNA preparation

Total RNA, poly [A+] RNA and first strand cDNA were prepared from *Manduca* eggs as above. To prevent adherence of the single stranded cDNA, all plastic and glassware was siliconised. The RNA strand of the RNA-cDNA hybrid was hydrolysed by incubation of the RNA-cDNA hybrid with an equal volume of 0.3M NaOH at 37°C for 2h and neutralised by dropwise addition of 1M HCl; the excess oligonucleotide, RoRi(dT)₁₇ removed by passage through a Sephacryl HR S-400 spin column and the single stranded cDNA purified once by phenol extraction and once by chloroform extraction.

2.3.2 Techniques used in the purification of nucleic acids

Extraction of nucleic acids with phenol:chloroform

Purification of nucleic acids by phenol:chloroform extraction was routinely performed as described in Sambrook *et al.* (1989).

Concentration of nucleic acids by ethanol precipitation

Nucleic acids were concentrated by precipitation with 0.1 volumes 3M sodium acetate, pH4.8 and 2.5 volumes absolute ethanol at -70°C for 20min or -20°C overnight, centrifuged at high speed for 20min, washed in 70% (v/v) ethanol and resuspended in sterile water.

Purification of DNA from agarose gels

Bands of DNA were excised from agarose gels and purified using a GeneClean® II kit (Bio101, Inc.) as described in the manufacturer's protocol.

Removal of short fragments of DNA

Fragments of DNA less than 100bp in size, were removed after enzymatic reactions, if necessary, using the High Pure PCR Product Purification Kit (Boehringer Mannheim) as described in the manufacturer's protocol.

2.3.3 Techniques used in the analysis of nucleic acids

Estimation of concentration of nucleic acid

The concentration of nucleic acid was calculated from readings of the absorbance at 260nm on a Perkin Elmer UV/VIS Lambda 11 Spectrometer. For double-stranded DNA,

at 260nm, 1OD = 50µg/ml; for single stranded DNA and RNA, at 260nm, 1OD = 40µg/ml and for single stranded oligonucleotides, at 260nm, 1OD = 20µg/ml (Sambrook *et al.*, 1989). Contamination with protein was calculated using the ratio: absorbance at 260nm / absorbance at 280nm.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described in Sambrook *et al.* (1989). DNA samples for agarose and low gelling temperature agarose contained 0.16 volumes of 6x loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll). These were loaded onto 1x TAE (see Appendix 3) gels of varying percentage agarose, containing 0.5µg/ml ethidium bromide. Gels were photographed under U.V. light using a Polaroid camera with yellow filter and Polaroid 667 black and white ISO 3000/36° film.

Radiolabelled cDNA samples for alkaline agarose gels contained 0.16 volumes of 6x alkaline loading buffer (300mM NaOH, 6mM EDTA, 18% (w/v) Ficoll, 0.15% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF). These were loaded onto 1% (w/v) agarose gels containing 50mM NaOH, 1mM EDTA, pH8.0. Gels were dried and autoradiographed at room temperature with Kodak X-Omat LS™ film.

Denaturing gels

All solutions were treated with 0.1% (v/v) DEPC at room temperature overnight and subsequently autoclaved to destroy RNase. RNA samples were denatured at 55°C for 1min in 1x formaldehyde buffer (40mM MOPS, 10mM sodium acetate, 1mM EDTA, pH8.0), 2.2M formaldehyde, 50% (v/v) formamide, then mixed with loading buffer (50% (v/v) glycerol, 1mM EDTA, pH8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) and loaded on 1% (w/v) agarose gels containing 1x formaldehyde running buffer and 2.2M formaldehyde. Gels were run at 90mV for 3h.

Sequencing

DNA was sequenced using the dideoxynucleotide chain termination method of Sanger *et al.* (1977), using T7 DNA Polymerase in a Sequenase™ Version 2.0 DNA Sequencing Kit (United States Biochemical/Amersham). Samples were electrophoresed on a 6% (w/v) acrylamide sequencing gel, for 2-4h at 30mA, 40W. Gels were fixed in 10% (v/v) methanol, 10% (v/v) acetic acid, dried and exposed to Kodak X-Omat LS™ film at room temperature overnight. A number of PCR products were sequenced directly on a Perkin Elmer ABI PRISM™ 377 DNA Sequencer.

2.3.4 Techniques used in the manipulation of nucleic acids

Restriction digests

Plasmid DNA digests

Plasmid DNA (up to 1 μ g) in sterile water, 0.1 volumes of the appropriate 10x reaction buffer and 1Unit/(μ g DNA) of the relevant restriction enzyme were mixed gently and incubated under optimal conditions of time and temperature (generally 37°C overnight). If necessary, 0.01 volumes of 100x BSA (10mg/ml) was also added to the digest. The digest was stopped by the addition of EDTA to 5mM and/or heat inactivation of the restriction enzyme (generally 75°C for 20min). DNA needing double digestion under incompatible conditions was digested with one restriction enzyme, the restriction enzyme inactivated, the DNA ethanol precipitated and then re-digested with the second restriction enzyme.

Genomic DNA digests

Genomic DNA (30 μ g), sterile water and 0.1 volumes of the appropriate 10x reaction buffer were incubated at 4°C for 4h. Restriction enzyme (30Units) was added, stirred gently and incubated at 37°C for 15min. Another 30Units of restriction enzyme was added, stirred and incubated at 37°C overnight. RNase (10Units) was also added to digest any remaining RNA.

Ligations

Ligations using T4 DNA Ligase

Ligations were performed with varying vector:insert ratios (generally a 3-fold molar excess of vector, a 3-fold molar excess of insert and equal amounts of vector and insert). An optimal amount of vector (250ng), the corresponding amount of insert, 0.2 volumes 5x T4 DNA Ligase buffer (250mM Tris-Cl, pH7.6, 50mM MgCl₂, 25% (w/v) PEG8000, 5mM ATP, 5mM DTT) and 5Units T4 DNA Ligase were mixed in a minimal volume.

Blunt end ligations were incubated at room temperature overnight whereas sticky end ligations were incubated at 16°C overnight. Ligations were diluted 5-fold in sterile water before adding to competent cells.

Ligations using T4 RNA ligase

Ligation of oligonucleotide to single stranded cDNA was achieved by incubation of 1-2pmol of a 5' phosphorylated, 3' blocked oligonucleotide and an aliquot of purified single stranded cDNA with 0.1 volumes of 10x T4 RNA Ligase buffer (500mM Tris-Cl, pH7.5,

100mM MgCl₂, 10mM hexamine cobalt chloride), 25% (w/v) PEG8000, 20μM ATP, 10μg/ml BSA and 20Units T4 RNA Ligase at 22°C for 18h.

Basic polymerase chain reactions (PCR)

PCR amplification of receptor plasmid DNA and cDNA fragments was performed using oligonucleotide primers designed to specific sequences within the receptor. PCR was performed in a 50μl total volume, containing 0.1 volumes 10x reaction buffer (500mM KCl, 100mM Tris-Cl, pH9.0, 1% (v/v) triton X-100), 1.5-4mM MgCl₂, 0.2mM dNTPs, 1μM each primer and approximately 100ng DNA template. If necessary, magnesium titrations were performed in order to achieve the optimal amount of specific product.

Using a Perkin Elmer Cetus DNA Thermal Cycler, the template was denatured at 95-96°C for 5min (an hotstart reaction), 2.5Units of *Taq* DNA Polymerase (Promega) was added and generally 30-40 cycles of denaturation at 95°C for 1min, annealing at 37-65°C for 1min and extension at 72°C for 1-2min commenced. The cycles were followed by a final extension of 72°C for 10min. Exact conditions were dependent on the annealing temperature and GC content of primer pairs (see calculation of annealing temperature in Appendix 2, part d) and the length of the template.

In some cases, the proof reading enzyme, Vent_R® DNA Polymerase (New England Biolabs) was used. Conditions were similar but used 10x reaction buffer containing 100mM KCl, 200mM Tris-Cl, pH8.8, 100mM (NH₄)₂SO₄, 20mM MgSO₄, 1% (v/v) Triton X-100.

PCR amplification of genomic DNA utilised the long template DNA polymerase system called Expand™ (Boehringer Mannheim). Genomic DNA (250ng), 0.1 volumes of 10x reaction buffer (buffer 1: 500mM Tris-Cl, pH9.2, 160mM (NH₄)₂SO₄, 17.5mM MgCl₂; buffer 2: 500mM Tris-Cl pH9.2, 160mM (NH₄)₂SO₄, 22.5mM MgCl₂; buffer 3: 500mM Tris-Cl, pH9.2, 160mM (NH₄)₂SO₄, 22.5mM MgCl₂, 20% (v/v) DMSO, 1% (v/v) Tween 20), 350μM or 500μM dNTPs for buffer 1 or buffers 2 and 3 respectively, 300nM sense primer and 300nM antisense primer and 2.5Units Expand™ were incubated at 94°C for 2min and then subjected to 10 cycles of 94°C for 10s, 55°C for 30s and 68°C for 12min, followed by 20 cycles of the same conditions but with an extra 20s for each extension step of each cycle. Any PCR products were subjected to a final extension of 68°C for 7min.

Subcloning PCR products

Primers incorporating unique restriction sites resulted in PCR products that were digested with the appropriate restriction enzyme, re-purified and ligated into digested pBluescript.

PCR primers that were unable to incorporate restriction sites resulted in PCR products that were phosphorylated and ligated into a blunt ended, dephosphorylated vector. Amplified DNA was phosphorylated with the addition of 0.1 volumes 10x buffer (300mM Tris-Cl, pH7.8, 100mM MgCl₂, 100mM DTT, 5mM ATP), 0.2mM dNTPs, Klenow (5Units) and T4 Polynucleotide Kinase (10Units), with incubation at room temperature for 2h. The enzymes were heat inactivated at 70°C for 10min and the DNA extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA was ethanol precipitated and resuspended in sterile water.

Vector (pBluescript) was digested with *EcoRV* at 37°C for 2h. Following heat inactivation of the enzyme at 75°C for 20min, the digested vector was dephosphorylated with 6µl calf intestinal alkaline phosphatase (CIAP, 1Unit/µl) in 10x CIAP buffer (500mM NaCl, 100mM Tris-Cl, pH7.9, 100mM MgCl₂, 10mM DTT) with incubation at 50°C for 1h. The enzyme was inactivated with EDTA to 5mM and heat at 75°C for 10min. The DNA was extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1), ethanol precipitated and resuspended in sterile water.

Vector and insert were mixed in varying ratios as described under Ligations using T4 DNA ligase.

2.3.5 Techniques used in radioactive hybridisation

Southern blots

DNA gels were rinsed in distilled water, incubated at room temperature with shaking for 30min in denaturation solution (1.5M NaCl, 0.5M NaOH), rinsed again in distilled water and incubated twice at room temperature with shaking for 15min each in neutralisation solution (1.5M NaCl, 0.5M Tris-Cl, pH7.2, 1mM EDTA) before being transferred to positively charged nylon membrane.

The gel was placed on a Whatman® 3MM filter paper wick soaked in 10x SSC (see Appendix 3) and each side edged with parafilm to stop horizontal diffusion. Nylon

membrane was placed on the gel, covered with Whatman® 3MM filter paper and paper towels and a heavy weight placed on top to allow transfer overnight.

DNA was fixed on the nylon membrane by baking at 80°C for 2h.

Radiolabelling of probes

Radiolabelled DNA probes were prepared using the ³²P QuickPrime™ Kit (Pharmacia) according to the manufacturer's protocol. Denatured DNA (50ng) was incubated with reaction mix, 50μCi [α -³²P]dCTP (specific activity 3000Ci/mmol; 10mCi/ml) and 4-8Units T7 DNA Polymerase at 37°C for 30min.

Purification of radiolabelled probes

Unincorporated radiolabel was removed from radiolabelled probes using MicroSpin™ columns (Pharmacia), generally of Sephacryl S-200 HR type, according to the manufacturer's protocol.

Hybridisation

Blots were soaked in 2x SSC at room temperature with shaking for 5min. Blots were prehybridised in hybridisation solution (50% (v/v) formamide, 6x SSC, 0.5% (w/v) SDS, 0.2% (w/v) sodium pyrophosphate, 5x Denhardt's solution (see Appendix 3), 0.1mg/ml denatured, sonicated, purified salmon sperm DNA) at 42°C, with shaking overnight. Purified, labelled probe was added to the blot in fresh hybridisation solution and hybridised with shaking at 42°C overnight. Blots were generally washed once in 2x SSC, 0.5% (w/v) SDS at room temperature for 5min, once in 2x SSC, 0.1% (w/v) SDS at room temperature for 15min and twice in 0.1x SSC, 0.5% (w/v) SDS at 37°C for 30min, although the exact stringency of washing was varied according to the level of background noise for each blot. Blots were exposed to Kodak X-Omat LS™ film with intensifying screens at -70°C overnight or longer.

Stripping the blot of radiolabelled probe

After each hybridisation, the blot was stripped of probe by incubation with shaking in 0.2M NaOH at room temperature for 20min, followed by incubation with shaking in neutralisation solution (0.2M Tris-Cl, pH7.8, 0.1x SSC, 0.1% (w/v) SDS), at 42°C for 2x 15min.

2.3.6 Techniques used in non-radioactive hybridisation for screening of a cDNA library

Plating out

E. coli XL-1 blue host cells were grown with shaking at 37°C overnight in NZY (see Appendix 3) supplemented with 1M MgSO₄ and 20% (w/v) maltose. The cells were spun down and resuspended in 10mM MgSO₄ to give an A₆₀₀ of 0.5.

Serial dilutions of the library in the bacteriophage vector, λZapII, were prepared in SM buffer (see Appendix 3). XL-1 blue cells (200μl) were mixed with dilutions of the library (100μl) and incubated at 37°C for 30min. Controls contained XL-1 blue cells and SM buffer. The library was spread onto warm 1.5% (w/v) NZY agar plates (large BioAssay dishes of dimensions 250x250mm) using 0.7% (w/v) NZY top agarose containing the infected host cells at a density of 150,000 pfu/plate. Plates were incubated at 37°C. Sequential screens were plated at a density of 500 pfu/plate (90mm culture plates) using bacteriophage λ eluted into SM buffer from plaques yielding a positive signal in the previous screen.

Plaque lifts

Duplicate plaque lifts, orientated by needle holes, were taken for each plate, the first for 1min and the second for 5min. The DNA was denatured in 1.5M NaCl, 0.5M NaOH for 5min and then neutralised in 0.5M Tris-Cl, pH7.4, 1.5M NaCl for 5min. The filters were washed in 2x SSC and fixed by baking at 80°C for 2h.

Probe preparation

Oligonucleotide 3' tailing

Oligonucleotides were labelled with Digoxigenin (DIG) -11-dUTP using a 3' tailing reaction. Oligonucleotide (5μM), was incubated with 1x reaction buffer (0.2M potassium cacodylate, 25mM Tris-Cl, pH6.6, 0.25mg/ml BSA), 5mM CoCl₂, 0.05mM DIG-11-dUTP, 0.5mM dATP and 2.5Units/μl Terminal Transferase at 37°C for 15min. Glycogen (0.02mg) and EDTA to 10mM were added and the labelled oligonucleotide precipitated with 0.1 volumes 4M LiCl and 3 volumes absolute ethanol at -70°C for 30min. Labelled oligonucleotide was centrifuged at high speed for 15min, washed with 70% (v/v) ethanol, dried and resuspended in 20μl sterile water.

Random prime labelling

DNA was labelled with DIG-11-dUTP using the random prime reaction. DNA (~150µg/ml) was incubated with 1x hexanucleotide mixture (6.25 A₂₆₀ units/ml random hexanucleotides, 50mM Tris-Cl, pH7.2, 10mM MgCl₂, 0.1mM Dithioerythritol, 0.2mg/ml BSA), 1x dNTP labelling mixture (0.1mM dATP, 0.1mM dCTP, 0.1mM dGTP, 0.065mM dTTP, 0.035mM DIG-11-dUTP, pH7.5), and 100Units/ml Klenow at 37°C for at least 1h. EDTA to 10mM was added and the labelled DNA precipitated as for oligonucleotide 3' tailing.

Hybridisation

Filters were prehybridised in hybridisation solution (5x SSC, 1% (w/v) blocking agent, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS) with shaking for 2h at 45°C or 68°C for oligonucleotide or random primed probe respectively. Oligonucleotide probes were diluted to 1pmol/ml in hybridisation solution with the addition of 100µg/ml poly(A). Filters probed with labelled oligonucleotides were hybridised at 45°C for 3h. Random primed probes were diluted to 5ng/ml in hybridisation solution. Filters probed with random prime labelled DNA were hybridised at 68°C overnight.

Washing

Washing of the filters was carried out twice in 2x SSC, 0.1% (w/v) SDS at room temperature for 5min each and twice in 0.1x SSC, 0.1% (w/v) SDS at the temperature of the hybridisation for 15min each.

Detection

Colourimetric detection using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) was carried out on oligonucleotide probed filters and random primed fragment c probed filters. All incubations were at room temperature with shaking. Filters were equilibrated in buffer 1 (100mM maleic acid, 150mM NaCl, pH7.5) for 1min and then blocked by shaking in buffer 2 (1% (w/v) blocking reagent in buffer 1) for 30min. Filters were incubated in Anti-DIG-alkaline phosphatase (150mU/ml) for 30min and unbound antibody removed by washing twice in buffer 1, for 15min each. After equilibration of the filters in buffer 3 (100mM Tris-Cl, pH9.5, 100mM NaCl, 50mM MgCl₂) for 2min, colour substrate (0.3mg/ml NBT and 0.17 mg/ml X-phosphate) was added and the filters incubated in the dark overnight. The reaction was stopped by washing the filters in buffer 1.

Chemiluminescent detection using Lumigen™ PPD [4-methoxy-4-(3-phosphate-phenyl)-spiro(1,2-dioxetane-3,2'-adamantane) disodium salt] was performed on oligonucleotide

probed filters and random primed DNA probed filters. Filters were incubated as above except Lumigen™ PPD (0.235mM) was substituted for colour substrate. Following a 5min incubation in Lumigen™ PPD, the filters were incubated at 37°C for 15min and exposed to Kodak X-Omat LS™ film at room temperature for 15min to overnight.

After detection, duplicated positive signals that corresponded to plaques, were picked from the corresponding plate and eluted into SM buffer.

2.3.7 Oligonucleotides

In house synthesis and deprotection of oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems 381A DNA Synthesiser using chemicals supplied by Cruachem, Glasgow, U.K. Oligonucleotides were deprotected with ammonia solution of Analar grade at 55°C for 6h. The ammonia was neutralised with glacial acetic acid and the oligonucleotide precipitated with absolute ethanol. The precipitated oligonucleotide was spun, washed, dried and resuspended in 1ml sterile water. The absorbance at 260nm was taken and the oligonucleotide diluted to a working stock solution of 20µM.

Radiolabelling of oligonucleotides

Oligonucleotides at 2µM were radiolabelled with 5µCi [γ -³²P]ATP (specific activity 3000Ci/mmol; 10mCi/ml) and 10Units T4 Polynucleotide Kinase in 0.1 volumes of 10x buffer (300mM Tris-Cl, pH7.8, 100mM MgCl₂, 100mM DTT, 5mM ATP) at 37°C for 30min. The enzyme was inactivated with EDTA, pH8.0 to 5mM and 1 volume of sequencing stop dye (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) xylene cyanol FF) was added. The radiolabelled oligonucleotide was heated at 80°C for 3min and electrophoresed on a 12% (w/v) acrylamide sequencing gel for 1h at 30mA, 40W. The gel was immediately exposed to Kodak X-Omat LS™ film at room temperature for 15-30min.

2.4 Results

2.4.1 Screening of the cDNA library

The first step in isolating both the missing N-terminus and a full-length clone of MARA1 and/or identifying any other α -like subunits, was a library screen using a cDNA library made from pupal *Manduca* brain.

2.4.1.1 Initial characterisation of the cDNA library

PCR on the Manduca library

In order to confirm the presence of an α -like subunit of the *Manduca* nAChR in the library, specific primers were designed from regions of the sequence from fragment c. The primer sense 1, with the sequence 5' AACAAATGCTGACGGCAACTTT 3' was designed to the predicted extracellular region (nucleotide positions 79-99 on fragment c) and the primer antisense 1, with the sequence 5' GGACAGCGATACCTTCTC 3' was designed to the putative second transmembrane domain (nucleotide positions 544-561 on fragment c) as shown in Appendix 2 parts a and c. These sequences were used as comparison with other insect α -like sequences (see alignment, Figure 2.36) indicated a high degree of homology between α -like subunits for these particular regions. Thus it was assumed, that if MARA1 was not found, another α -like subunit might be present. PCR as described in section 2.3.4, resulted in the amplification of a fragment of approximately the expected size of 483bp (Figure 2.1), suggesting that an α -like subunit of the nAChR probably corresponding to the sequence of MARA1 was present in the library.

Calculating the library titre

In order to determine the density of plaques needed for screening, the library titre was measured by plating out the library, as described in section 2.3.6. The titre of the cDNA library was calculated to be 5.76×10^9 pfu/ml.

Determining the range of insert size

In order to assess the range of insert size, plaques were picked at random and used in PCR with the pBluescript primers T3 (sense) and T7 (antisense) (sequences: 5' ATTAACCCTCACTAAAG 3' and 5' AATACGACTCACTATAG 3' respectively). PCR resulted in insert sizes ranging from 500bp to 2000bp as shown in Figure 2.2, indicating the presence of relatively large cDNA clones in the library.

2.4.1.2 Results of the cDNA library screen

Identification of positive plaques

The Digoxigenin (DIG) non-radioactive method was used to screen the *Manduca* cDNA library, in order to find a full-length cDNA encoding MARA1. Primers specific to the MARA1 sequence (sense 1 at nucleotide positions 79-99 of fragment c and antisense 1 at positions 544-561 of fragment c; see Appendix 2, parts a and c) were labelled with DIG-11-dUTP by the 3' tailing technique. The efficiency of labelling was checked by colour detection and this revealed detection at 4fmol/ μ l. Consequently, both primers were used in separate solutions for hybridisation to plaque lifts. Colourimetric detection was applied to the lifts using NBT and X-phosphate. The primary screen resulted in a number of positive signals, of which only one was present in duplicate. The plaques were picked from the corresponding region on the plate and taken through a secondary screen which did not result in any positive signals.

The partial length cDNA, fragment c, was labelled with DIG-11-dUTP using the random prime technique. The efficiency of labelling was checked by colour detection. Fragment c was detected at a concentration of 25pg/ μ l. The library was screened again using the random primed fragment c and colour detection but did not result in any positive signals.

The labelling efficiency of the random primed fragment c was then assessed by chemiluminescent detection and fragment c was detected at a concentration of 0.002pg/ μ l. The library screen was repeated again. A total of 900,000 plaques were screened using random primed fragment c and chemiluminescent detection. Several plaques corresponding to positive signals were picked from the primary screen, however these positive signals were not in duplicate. Sequential screens did not result in any duplicated signals.

Approximately 1.5 million plaques were screened in total and no duplicated positives were found. A control using fragment c dotted onto a membrane was successful, thus indicating that a technically correct labelling and detection procedure was being applied. A number of variables, such as altering the hybridisation temperature and also the stringency during washing were altered but these did not affect the outcome.

Nested PCR on the Manduca cDNA library

In order to facilitate finding the full-length cDNA, nested PCR on the library was performed. For the missing N-terminus to be amplified, primary and secondary reactions

utilised primers designed from fragment c and from the library vector λ ZapII, as shown in Figure 2.3.

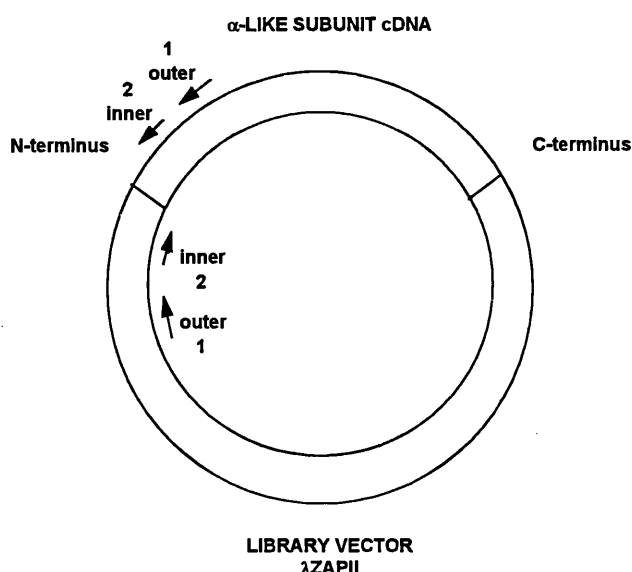


Figure 2.3. Nested PCR on *Manduca* cDNA library. (Not to scale).

1. Primary round of amplification with outer primers (1) designed from λ ZapII (sense) and fragment c (antisense 1, see Appendix 2), using 35 cycles of 95°C for 1min, 45°C for 1min and 72°C for 2min.
2. Product diluted 1 in 100 in sterile water.
3. Secondary round of amplification with inner pair of primers (2) designed from λ ZapII (sense T3) and fragment c (antisense 2, see Appendix 2), using 35 cycles of 95°C for 1min, 55°C for 1min and 72°C for 2min.

The outer pair of primers designed from λ ZapII (sense), with the sequence 5' AACAGCTATGACCATG 3', and fragment c (antisense 1 at nucleotide positions 544-561 of fragment c; see Appendix 2, parts a and c) with the sequence 5' GGACAGCGATACCTTCTC 3' were used in a primary amplification, the product from which was diluted 1 in 100 in sterile water and subjected to a secondary amplification with the inner pair of primers, from λ ZapII, T3 (sense) with the sequence 5' ATTAACCCTCACTAAAG 3' and from fragment c (antisense 2 at positions 313-336 of fragment c; see Appendix 2, parts a and c) with the sequence 5' AAACCTCGGAGAGGTCGACGCCCAA 3'. Amplification of the missing N-terminus from the cDNA library resulted in a fragment of approximately 600bp that was smaller than the band of approximately 700bp (calculated size: 697bp) for the positive control, fragment c in the primary round and in a fragment of approximately 400bp (calculated size: 421bp) in the secondary round which was the same size as the band for the positive control, fragment c (Figure 2.4). Although, this cDNA was not sequenced, the results suggested that the library contained only truncated cDNA encoding a partial length α -like subunit corresponding to MARA1 and consequently was of no use.

As the library screen did not result in additional sequence information, methods based on the polymerase chain reaction were attempted.

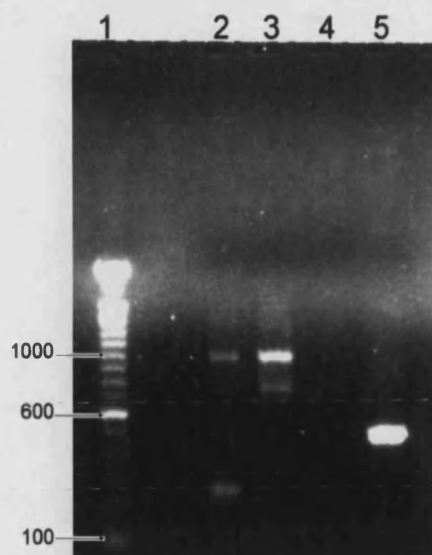


Figure 2.1. PCR on the *Manduca* cDNA library to confirm the presence of an α -like subunit in the library. cDNA, PCR reaction mix (as described in section 2.3.4) and primers (described in section 2.4.1.1) were incubated at 95°C for 5min, 2.5Units of *Taq* DNA Polymerase was added and the mix subjected to 35 cycles of 95°C for 1min, 48°C for 1min and 72°C for 2min. Products were analysed on a 1% (w/v) agarose gel. Lane 1, 100bp ladder (sizes in bp); lane 2, library cDNA amplified with both sense and antisense primers; lane 3, negative control of library cDNA amplified with sense primer only; lane 4, negative control of library cDNA amplified with antisense primer only; lane 5, positive control of fragment c amplified with both sense and antisense primers. Lane 2 shows faint band of ~500bp corresponding to band amplified in positive control.

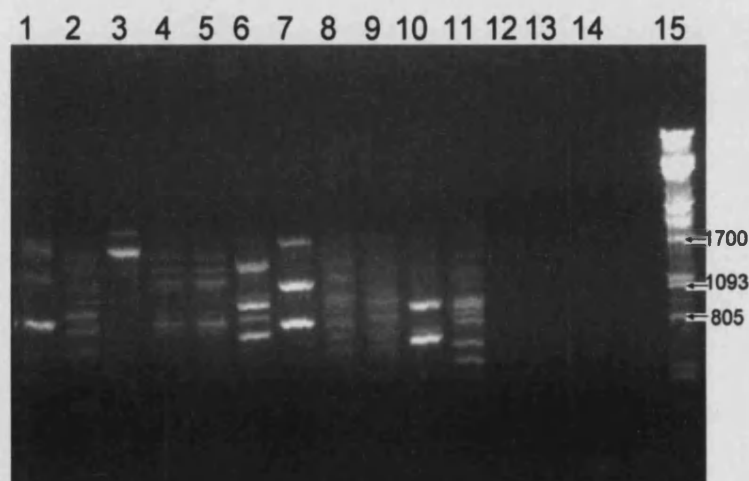


Figure 2.2. PCR on randomly picked plaques from the library screen to determine range of insert size. Plaques, PCR reaction mix (as described in section 2.3.4) and primers (described in section 2.4.1.1) were incubated at 95°C for 5min, 2.5Units of *Taq* DNA Polymerase was added and the mix subjected to 30 cycles of 95°C for 1min, 55°C for 1min and 72°C for 1min. Products were analysed on a 1% (w/v) agarose gel. Lanes 1-10 show PCR products ranging from 500bp to 2000bp; lanes

11-13, negative controls; lane 14, positive control of fragment c in pBluescript, expected size of band ~1600bp which was not revealed on the gel; lane 15, λ DNA digested with *Pst*I (sizes in bp).

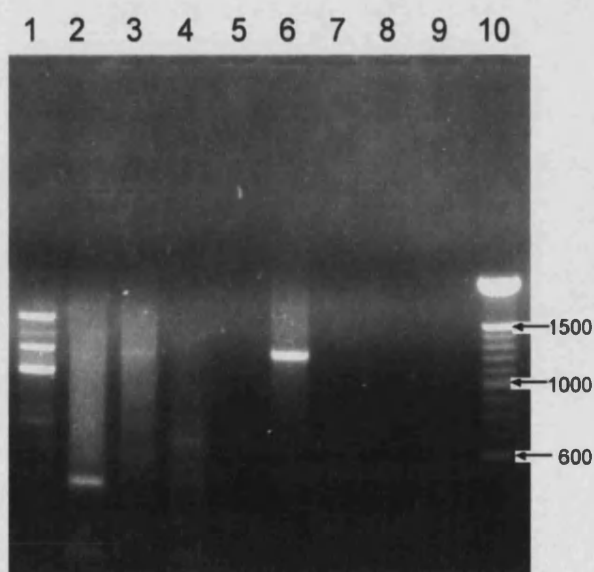


Figure 2.4. Nested PCR on the *Manduca* cDNA library. The primary round of amplification with outer primers designed from λ ZapII (sense) and fragment c (antisense 1, see Appendix 2), used 35 cycles of 95°C for 1min, 45°C for 1min and 72°C for 2min. The resulting PCR product was diluted 1 in 100 in sterile water and used in a secondary round of amplification. The secondary round of amplification with inner pair of primers designed from λ ZapII (sense T3) and fragment c (antisense 2, see Appendix 2), used 35 cycles of 95°C for 1min, 55°C for 1min and 72°C for 2min. Products were analysed on a 1% (w/v) agarose gel. Lanes 1 and 2, positive controls using fragment c in outer and inner rounds of amplification; lanes 3 and 4 library cDNA amplified with both sense and antisense primers in outer and inner rounds of amplification; lanes 5-9, negative controls (5, no cDNA; 6-9, either sense or antisense primer with library cDNA only, outer followed by inner rounds of amplification); lane 10,

100bp ladder (sizes in bp). Lane 3 (experimental) shows band ~100bp smaller than that from positive control (lane 1), suggesting the presence of truncated cDNA.

2.4.2 Inverse PCR on double stranded, circularised cDNA

A PCR based strategy which used inverse PCR on double stranded, circularised cDNA (Towner and Gärtner, 1992) was attempted. This involved the isolation of total RNA and poly [A+] RNA from *Manduca*, the latter subsequently being used for the synthesis of first strand cDNA. This in turn, was used to synthesise double stranded cDNA which was circularised and used as a template in inverse PCR. This inverse PCR strategy is shown in Figure 2.5. RNA was isolated from day 2-3 eggs, as transcription levels of nAChRs in insects are particularly high in late embryos and in late pupae, i.e. during periods of terminal differentiation and synapse formation in the CNS (Sanes *et al.*, 1977; Hildebrand *et al.*, 1979; Hermans-Borgmeyer *et al.*, 1986). Also, eggs were used rather than pupae due to ease of collection.

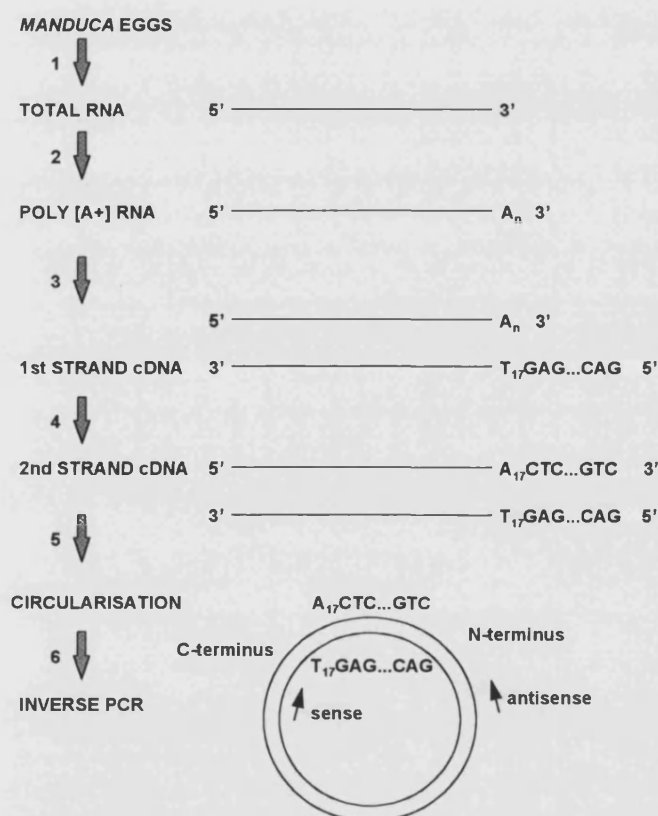


Figure 2.5. Inverse PCR on double stranded, circularised cDNA (adapted from Towner and Gärtner, 1992). (Not to scale).

1. Guanidinium thiocyanate phenol chloroform extraction of total RNA (see section 2.3.1).
2. Dynabead® extraction of poly [A+] RNA (see section 2.3.1).
3. 1st strand cDNA synthesis using SuperScript™ II RNase H- RT (see section 2.3.1).
4. 2nd strand cDNA synthesis using DNA Polymerase I; RNA degradation using RNase H (see section 2.3.1).
5. Formation of blunt ends using T4 DNA Polymerase; circularisation using T4 DNA Ligase (see section 2.3.1).
6. Inverse PCR using primer sense 2 (see Appendix 2) to 3' end and primer antisense 3 (see Appendix 2) to 5' end.

cDNA, PCR reaction mix (see section 2.3.4) and primers were incubated at 95°C for 5min, 2.5Units of *Taq* DNA Polymerase added and 30 cycles of 95°C for 1min, 55°C for 1min and 72°C for 1min were completed.

For inverse PCR, a sense primer was designed from the 3' end of fragment c shortly before the poly [A+] tail (sense 2 at nucleotide positions 1601-1624 of fragment c; see

Appendix 2, parts a and c) and an antisense primer was designed from the 5' sequence of fragment c shortly before the N-terminus (antisense 3 at nucleotide positions 67-90 of fragment c; see Appendix 2, parts a and c). The primer sequences were, sense 2, 5' ACGAAATAAAATGATCGTTTGTAT 3' and antisense 3, 5' GTCAGCATTGTTGTAAAGCACGAT 3'.

2.4.2.1 Analysis of preparative steps and inverse PCR products

The total RNA isolated was analysed on a denaturing gel (see section 2.3.3) which revealed one bright band of rRNA (RNA markers indicated that the *Manduca* rRNA was between 1.35 and 2.37 kb) as normally found in *Manduca* (Fujiwara *et al.*, 1995; A.Wang - personal communication). The concentration of total RNA was 1.5mg/ml, with an A_{260}/A_{280} of 1.85.

Analysis of first strand synthesis by incorporation of radiolabel showed first strand cDNA ranging in size from 500bp to 7kb (Figure 2.6).

Analysis of the inverse PCR by agarose gel electrophoresis and ethidium bromide staining (see section 2.3.3) did not reveal any bands of the anticipated size (300bp) as estimated from comparison of fragment c with full-length α -like subunits from other insects (see alignment, Figure 2.36). In an attempt to isolate the 5' end, a slice of the gel that would contain DNA products ranging in size from 200-400bp was excised from the gel. This DNA was purified with GeneClean® II and ethanol precipitated at -20°C overnight as described in section 2.3.2. After resuspending the DNA in 10µl sterile water, the DNA was re-amplified in a PCR using the same sense and antisense primers but with 40 cycles. This resulted in a band of DNA of approximately 200bp as shown in Figure 2.7 which was excised, purified and subcloned into pBluescript for sequencing, as described in section 2.3.4.

2.4.2.2 Sequencing analysis of inverse PCR product

The nucleotide and corresponding amino acid sequence results for the inverse PCR product are shown in Figure 2.8. Analysis of the sequence data using the gcg program Fasta (Pearson and Lipman, 1988) showed high sequence identity between the inverse PCR product and insect α -like subunits of the nAChR (Figure 2.9) indicating that the inverse PCR product was an α -like subunit of the nAChR. Hereinafter, this inverse PCR product is called fragment b.

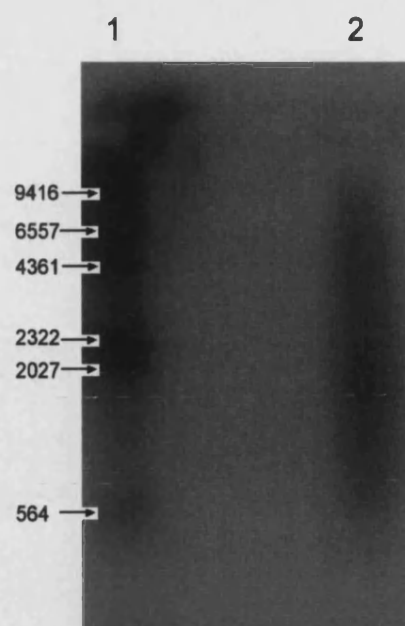


Figure 2.6. Analysis of first strand cDNA synthesised from RNA isolated from *Manduca* eggs. 1 μ l [α - 32 P]dCTP was added to 0.1 volumes of the total 1st strand cDNA reaction volume and incubated at 37°C for 1h. The cDNA was run on an alkaline agarose gel and exposed to Kodak X-Omat LS™ film. Lane 1, λ DNA digested with *Hind*III (sizes in bp); lane 2, 1st strand cDNA ranging in size from 500bp to 7kb.

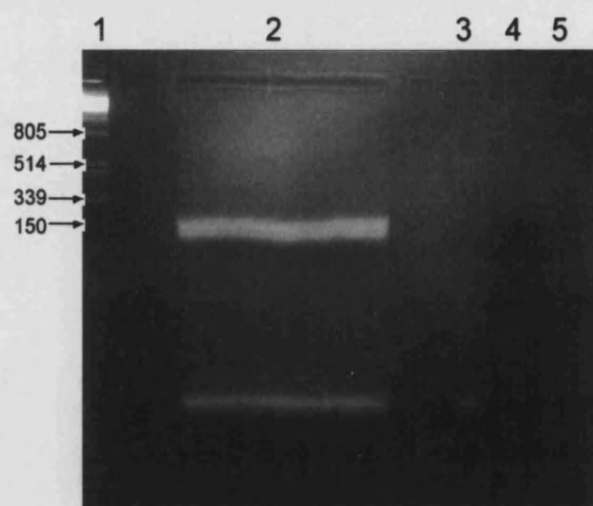


Figure 2.7. Inverse PCR on double stranded, circularised cDNA. cDNA, PCR reaction mix (as described in section 2.3.4) and primers sense 2 and antisense 3 (see Appendix 2) were incubated at 95°C for 5min, 2.5Units of *Taq* DNA Polymerase added and 40 cycles of 95°C for 1min, 55°C for 1min and 72°C for 1min were completed. Products were analysed on a 1% (w/v) agarose gel. Lane 1, λ DNA digested with *Pst*I (sizes in bp); lane 2, double stranded, circularised cDNA amplified with both primers, lanes 3-5, negative controls (3, no cDNA; 4 and 5, one primer only). Lane 2 shows band of ~200bp.

| | | |
|-----|--|-----|
| 1 | ACGAAATAAAATGATCGTTTGTATAATATTGCAAATTCGAATTTGCGTGAATCGAAAATA | 60 |
| 61 | CGAGCGAGCTGAGCCAGCTCATCGACGTGAATCTCAAGAATCAAATAATGACAACCAATC | 120 |
| 37 | L S Q L I D V N L K N Q I M T T N L | 54 |
| 121 | TATGGGTGGAACAGAGCTGGTACGACTACAAGCTTTCGTGGGAGCCGCGCGAGTACGGCG | 180 |
| 55 | W V E Q S W Y D Y K L S W <u>E P R E Y G G</u> | 74 |
| 181 | GCGTGGAATGCTGCACGTGCCTTCCGACCACATCTGGCGGCCCGACATCGTGCTTTACA | 240 |
| 75 | <u>V E M L H V P S D H I W R P D I V L Y N</u> | 94 |
| 241 | ACAATGCTGA | 250 |
| 95 | <u>N A D</u> | 97 |

Figure 2.8. Nucleotide and amino acid sequence of fragment b. Amino acids that are underlined represent overlap with fragment c. PCR artifact in italics (see text).

Fragment b is 250bp in length. The first 24bp of fragment b denote the sequence for the primer sense 2, however, the following 44bp appear to be a PCR artifact. Logically, any sequence appearing after the sense primer should be poly [A] tail, as the poly [A] tail signal cleavage site, AATAAA is present 20 nucleotides upstream from the poly [A] tail in fragment c (see Appendix 2). Possibly, this sequence is due to mis-ligation of the blunt ended double stranded cDNA during circularisation. The coding sequence representing the α -like subunit is 182bp (61 amino acid residues) long, of which 93bp (31 amino acid residues) are novel data as fragment b overlaps with fragment c, the original partial length sequence by 89bp (30 amino acid residues). This expansive overlap supports the assumption that fragment b and fragment c are part of the same α -like subunit, namely MARA1. Furthermore, this strongly suggests that this α -like subunit of the nAChR in *Manduca* is found in both the embryonic and pupal stages, justifying the decision to use material from *Manduca* eggs (the embryonic stage) for the inverse PCR strategy whilst material from the pupal stage had been used to isolate fragment c and was used in the library screen above. Additional sequencing (throughout regions of the sequence only discernable as compressions on a sequencing gel) replaced the initial amino acid residue in fragment c, an arginine (Clarke, 1991), with a glutamate.

Despite sequencing five more clones in both directions, no further 5' sequence could be isolated. Although some of the missing N-terminus of MARA1 had been found, an estimated 171-231bp (or 57-77 amino acid residues) by comparison with *Drosophila* and *Schistocerca* α -like subunits remained elusive (see alignment, Figure 2.36).

1) *S. gregaria* mRNA for neuronal nicotinic acetylcholine receptor (α L1/ARL2)
 ACCESSION NUMBER X55439
 74.7% identity in 182 bp overlap

| | | | | | | |
|--------|--|-----|-----|-----|-----|-----|
| | 40 | 50 | 60 | 70 | 80 | 90 |
| frag b | GAATTTGCGTGAATCGAAAATACGAGCGAGCTGAGCCAGCTCATCGACGTGAATCTCAAG | | | | | |
| | | | | | | |
| ARL2 | GACACGGTGCTCGTCAAGCTGGGCCTGCGCCTGTCGCAGCTCATCGACCTGAATCTGAAG | | | | | |
| | 170 | 180 | 190 | 200 | 210 | 220 |

| | | | | | | |
|--------|---|-----|-----|-----|-----|-----|
| | 100 | 110 | 120 | 130 | 140 | 150 |
| frag b | AATCAAATAATGACAACCAATCTATGGGTGGAACAGAGCTGGTACGACTACAAGCTTTTCG | | | | | |
| | | | | | | |
| ARL2 | GATCAGATCCTTACCACCAACGTCTGGTTGGAGCACGAATGGCAGGACCACAAGTTCCGA | | | | | |
| | 230 | 240 | 250 | 260 | 270 | 280 |

| | | | | | | |
|--------|---|-----|-----|-----|-----|-----|
| | 160 | 170 | 180 | 190 | 200 | 210 |
| frag b | TGGGAGCCGCGCGAGTACGGCGGCGTGGAATGCTGCACGTGCCTTCCGACCACATCTGG | | | | | |
| | | | | | | |
| ARL2 | TGGGACCCGCGGAGTACGGAGGCGTGACGGAGCTGTACGTCCCTTCGGAGCACATCTGG | | | | | |
| | 290 | 300 | 310 | 320 | 330 | 340 |

| | | | | |
|--------|---|-----|-----|-----|
| | 220 | 230 | 240 | 250 |
| frag b | CGGCCCCGACATCGTGCTTTACAACAATGCTGA | | | |
| | | | | |
| ARL2 | CTGCCCCGACATCGTCCTGTACAACAACGCTGACGGCGAGTACGTGGTCACGACCATGACT | | | |
| | 350 | 360 | 370 | 380 |

2) *D. melanogaster* D α 2 mRNA for nicotinic acetylcholine receptor (D α 2/SAD)
 ACCESSION NUMBER X52274
 72.7% identity in 183 bp overlap

| | | | | | | |
|--------|--|-----|-----|-----|-----|-----|
| | 40 | 50 | 60 | 70 | 80 | 90 |
| frag b | CGAATTTGCGTGAATCGAAAATACGAGCGAGCTGAGCCAGCTCATCGACGTGAATCTCAA | | | | | |
| | | | | | | |
| SAD | GGACACGGTGTTGGTCAAATTGGGCCTACGGCTCTCCCAACTCATCGATTTGAATCTCAA | | | | | |
| | 700 | 710 | 720 | 730 | 740 | 750 |

| | | | | | | |
|--------|--|-----|-----|-----|-----|-----|
| | 100 | 110 | 120 | 130 | 140 | 150 |
| frag b | GAATCAAATAATGACAACCAATCTATGGGTGGAACAGAGCTGGTACGACTACAAGCTTTC | | | | | |
| | | | | | | |
| SAD | AGATCAAATTCTAACCACCAACGTGTGGCTGGAGCACGAATGGCAGGATCATAAATTCAA | | | | | |
| | 760 | 770 | 780 | 790 | 800 | 810 |

| | | | | | | |
|--------|--|-----|-----|-----|-----|-----|
| | 160 | 170 | 180 | 190 | 200 | 210 |
| frag b | GTGGGAGCCGCGCGAGTACGGCGGCGTGGAATGCTGCACGTGCCTTCCGACCACATCTG | | | | | |
| | | | | | | |
| SAD | GTGGGATCCCTCGGAGTATGGCGGCGTCACAGAGCTTTATGTGCCATCCGAGCACATCTG | | | | | |
| | 820 | 830 | 840 | 850 | 860 | 870 |

| | | | | |
|--------|---|-----|-----|-----|
| | 220 | 230 | 240 | 250 |
| frag b | GCGGCCCCGACATCGTGCTTTACAACAATGCTGA | | | |
| | | | | |
| SAD | GCTGCCCCGACATCGTGCTCTACAACAATGCCGATGGCGAGTACGTGGTCACCACCATGAC | | | |
| | 880 | 890 | 900 | 910 |

Figure 2.9. Alignment of part of fragment b with part of the sequences encoding α L1 (ARL2) from *Schistocerca* and D α 2/SAD from *Drosophila*. These two sequences represent the highest nucleotide identities obtained from searching the Genbank and EMBL databases using the gcg program Fasta (Pearson and Lipman, 1988).

2.4.3 Ligation anchored PCR

A second PCR based method utilising a modification of the RACE method (rapid amplification of cDNA ends; see section 2.1; Frohman *et al.*, 1988) and termed ligation anchored PCR was attempted. In this a single stranded oligonucleotide (the anchor) was ligated to the 3' end of the first strand cDNA, with T4 RNA Ligase. A ligation anchored PCR based on a combination of methods adapted from Tessier *et al.* (1986), Edwards *et al.* (1991) and Troutt *et al.* (1992) was used. The ligation anchored PCR strategy is shown in Figure 2.10.

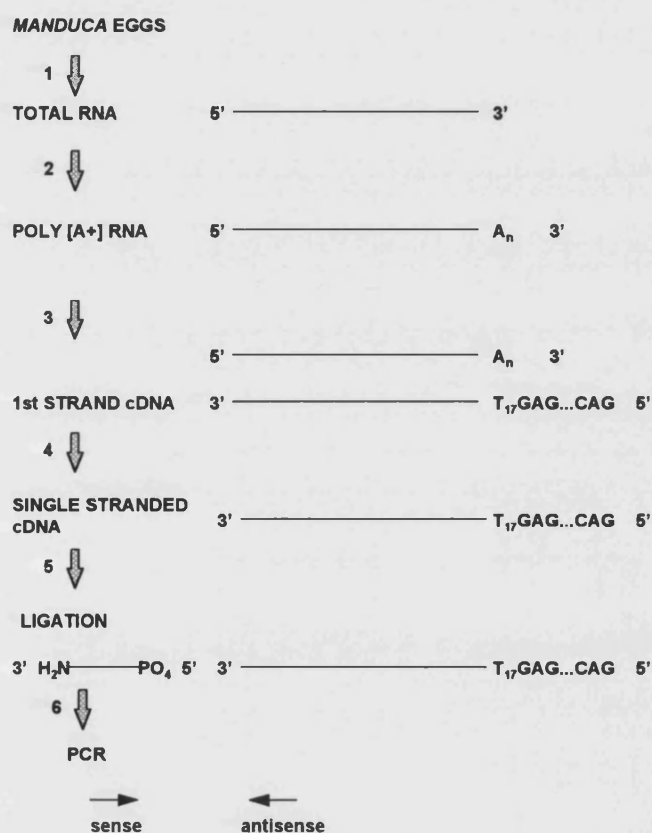


Figure 2.10. Ligation anchored PCR on first strand cDNA. (Not to scale).

1. Guanidinium thiocyanate phenol chloroform extraction of total RNA (see section 2.3.1).
2. Dynabead® extraction of poly [A+] RNA (see section 2.3.1).
3. 1st strand cDNA synthesis using SuperScript™ II RNase H- RT (see section 2.3.1).
4. Degradation of RNA with NaOH; removal of excess oligo(dT) with Sephacryl spin column (see section 2.3.1).
5. Ligation of anchor oligonucleotide to single stranded cDNA using T4 RNA Ligase (see section 2.3.4).
6. PCR using sense primer specific to anchor and primer antisense 4 specific to fragments b and c (see Appendix 2). cDNA, PCR reaction mix (see section 2.3.4) and primers were incubated at 95°C for 5min,

2.5Units of *Taq* DNA Polymerase added and 40 cycles of 95°C for 1min, 55°C for 1min and 72°C for 1min were completed.

The anchor oligonucleotide was designed to incorporate a T3 promoter sequence for direct *in vitro* transcription of the PCR product and the restriction sites *Not*I and *Eco*RI for easy cloning of the PCR product. The anchor oligonucleotide sequence (5' TTTAGTGAGGGTTAATAAGCGGCCGCACGTGAATTCGTACCTAC 3') was analysed for homology with other sequences using the Genbank and EMBL databases on the gcg program Fasta (Pearson and Lipman, 1988), it being imperative to avoid sequences that might anneal with the cDNA. The anchor oligonucleotide was synthesised with a

phosphoramidite group blocking the 3' end and checked by radiolabelling. After HPLC purification, 100pmol of anchor oligonucleotide was phosphorylated by incubation with 0.1 volumes of 10x T4 Polynucleotide Kinase buffer, 1mM ATP and 20Units T4 Polynucleotide Kinase at 37°C for 30min, in order for the anchor oligonucleotide to be ligated to the single stranded cDNA as described in sections 2.3.1 and 2.3.4.

PCR on the ligated anchor oligonucleotide and the single stranded cDNA used a sense primer to the anchor oligonucleotide (5' GCGGCCGCTTATTAACCCTCACTAAA 3') and the primer antisense 4 (5' TGTCATTATTTGATTCTTGAGATTCAC 3'; nucleotide positions 226-228+340-363 of fragment a, see Appendix 2, parts b and c) or the same sense primer and antisense 3 (5' GTCAGCATTGTTGTAAAGCACGAT 3'; nucleotide positions 67-90 on fragment c, see Appendix 2, parts a and c).

Agarose gel analysis of the PCR products, as shown in Figure 2.11 did not result in any bands of the appropriate size, i.e. approximately 242-302bp for the anchor sense primer and antisense 4 primer and approximately 380-440bp for the anchor sense primer and antisense 3 primer, by comparison with sequence data from other insect α -like subunits (see alignment, Figure 2.36).

Following the dearth of sequence data obtained using PCR methods involving the synthesis of cDNA, an approach utilising genomic DNA was attempted.

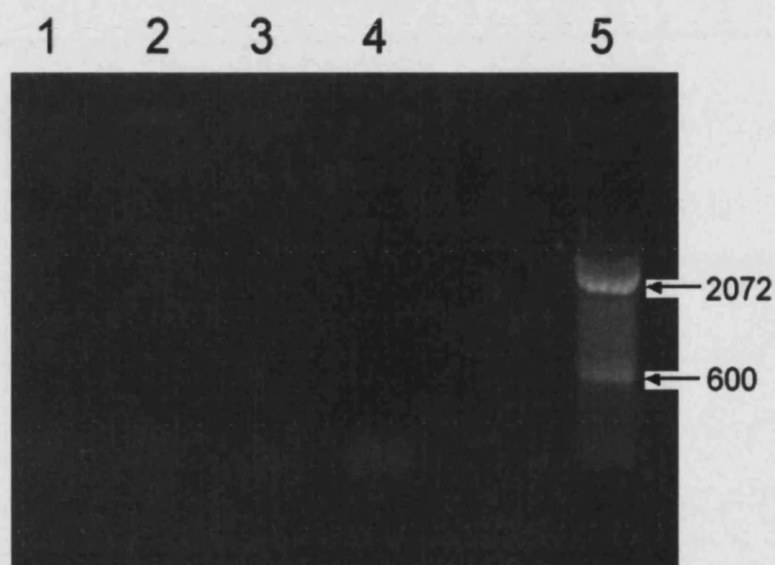


Figure 2.11. Ligation anchored PCR. PCR using sense primer specific to anchor and primer antisense 4 specific to fragments b and c (see Appendix 2). cDNA, PCR reaction mix (see section 2.3.4) and primers were incubated at 95°C for 5min, 2.5Units of *Taq* DNA Polymerase added and 40 cycles of 95°C for 1min, 55°C for 1min and 72°C for 1min were completed. Lane 1, ligation mix amplified with both sense and antisense primer; lanes 2, 3 and 4, negative controls (no cDNA, one primer only); lane 5, 100bp ladder (sizes in bp). No bands in lane 1 are visible.

2.4.4 Inverse PCR on circularised, genomic DNA

An inverse PCR strategy on genomic DNA was attempted using a method adapted from Forster *et al.* (1994). This is shown in Figure 2.12. Succinctly, genomic DNA was digested with restriction enzymes that had only one restriction site in the N-terminal of fragments b and c. DNA fragments which hybridised to a partial fragment from the 5' end of fragment c, indicating restriction sites in the genomic DNA within a relatively short distance of the current 5' end (i.e. within 3kb), were circularised by self ligation and used in an inverse PCR, to amplify the missing fragment.

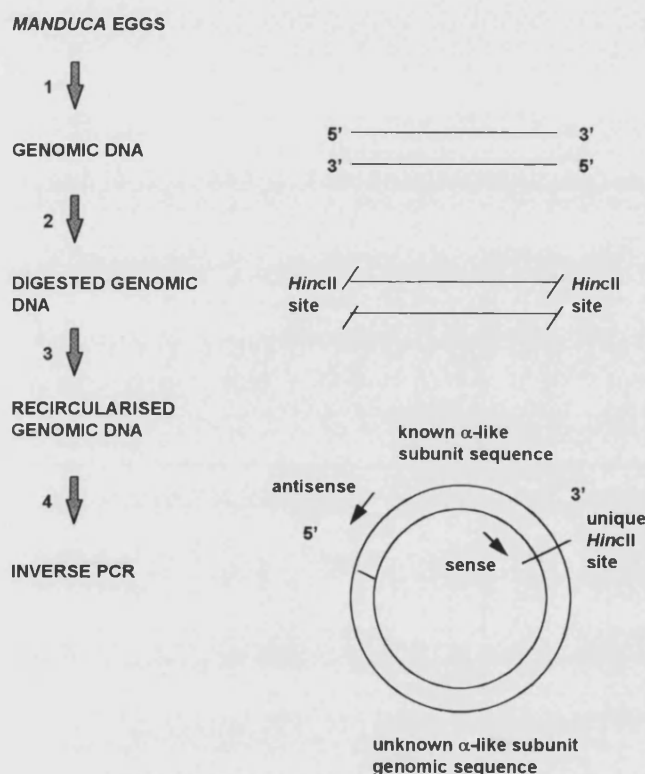


Figure 2.12. Inverse PCR on genomic DNA. (Not to scale).

1. Preparation of genomic DNA (see section 2.3.1).
2. Genomic DNA digested with *HincII* restriction enzyme (see section 2.3.4).
3. Circularisation using T4 DNA Ligase.
4. Inverse PCR using primer sense 4 to sequence shortly before unique *HincII* restriction site and primer antisense 3 to 5' end of fragment c (see Appendix 2). Genomic DNA (2μl), PCR reaction mix (see section 2.3.4) and primers were incubated at 95°C for 5min, 2.5Units of *Taq* DNA Polymerase added and 35 cycles of 95°C for 1min, 55°C for 1min and 72°C for 1min were completed.

2.4.4.1 Analysis of the genomic DNA

The genomic DNA was analysed on a 0.5% (w/v) agarose gel with a 1% (w/v) support and the A_{260}/A_{280} taken. The genomic DNA concentration was calculated to be 0.5μg/μl.

Restriction digests on the genomic DNA using a number of enzymes that cut near to the N-terminal of fragments b and c were performed in order to deduce which enzyme would be most appropriate for the subsequent digestion and circularisation of the genomic DNA. The enzymes *ScaI*, *BglII*, *XhoI*, *HincII* and *XbaI* were used. Control digests using

rat genomic DNA were included. Samples were ethanol precipitated and resuspended in 20µl sterile water. The digests were analysed on a large 0.6% (w/v) agarose gel, as shown in Figure 2.13, the DNA denatured and transferred to positively charged nylon membrane by Southern blotting as described in section 2.3.5. The probe (a partial length fragment from the 5' end of fragment c, excised from fragment c at the unique *Xba*I restriction site) was prepared and labelled with [α -³²P]dCTP and the blot prehybridised, hybridised, washed and exposed to film as described in section 2.3.5.

Bands between 2kb and 6kb were observed in lanes with *Manduca* genomic DNA digested with *Bgl*II, *Xho*II, *Hinc*II and *Xba*I as shown in Figure 2.14. Generally, this indicated restriction sites within approximately 4kb of the 5' end of fragments b and c. For the rat genomic DNA control, only DNA digested with *Hind*III produced an obvious band.

2.4.4.2 Inverse PCR on digested, circularised genomic DNA

Each of the successful enzymes (40Units) were used in repeat digestions of genomic DNA (20µg), as above. The digested DNA was ethanol precipitated and the washed, dried DNA resuspended in 792µl sterile water. The digested genomic DNA was circularised as described in section 2.3.1, Second strand cDNA synthesis and circularisation.

The primer antisense 3, (at nucleotide positions 67-90 of fragment c; see Appendix 2, parts a and c) which had been previously designed to the 5' end of fragment c (sequence 5' GTCAGCATTGTTGTAAAGCACGAT 3') and a different sense primer for each digestion, corresponding to a sequence shortly before the unique restriction site were used in inverse PCR on the genomic DNA. These were for *Xba*I, sense 3 (sequence 5' AAGTTCTACACGTGCTGCGAC 3'; at nucleotide positions 388-408 of fragment c; see Appendix 2, parts a and c), for *Hinc*II, sense 4 (sequence 5' GTGCGAGGCACCAACGTCGTG 3'; at nucleotide positions 289-309 of fragment c; see Appendix 2, parts a and c) and for *Xho*II/*Bgl*II sense 5 (sequence 5' AAGTTTGGCTCGTGGACCTAC 3'; at nucleotide positions 232-253 of fragment c; see Appendix 2, parts a and c). PCR resulted in bands on an agarose gel for the *Xba*I and *Hinc*II circularisations of approximately 200bp and 600bp respectively (the result using *Hinc*II is shown in Figure 2.15). The bands were excised, purified and subcloned into pBluescript as described in section 2.3.4.

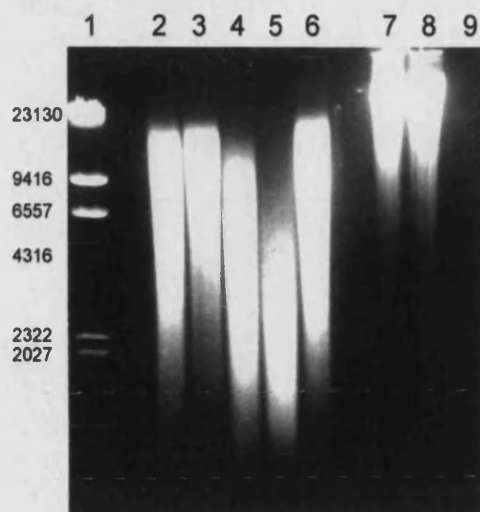


Figure 2.13. Genomic DNA digests. Genomic DNA from *Manduca* was digested with restriction enzymes and the products analysed on a 0.6% (w/v) agarose gel. Lane 1, λ DNA digested with *HindIII* (sizes in bp), lanes 2-6, *Manduca* genomic DNA digested with *Scal*, *BglII*, *XhoI*, *HincII* and *XbaI* respectively, lanes 7-9, rat genomic DNA digested with *HindIII*, *EcoRI* and *PstI* respectively.

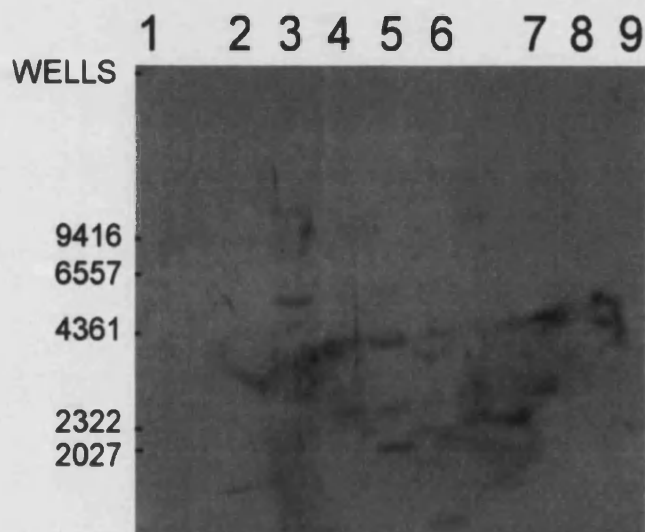


Figure 2.14. Southern blot of *Manduca* genomic DNA. Genomic DNA digests were run on an agarose gel, Southern blotted and the blot hybridised with a radiolabelled probe of fragment c (see text). Lane 1, λ DNA digested with *HindIII* (sizes in bp), lanes 2-6, *Manduca* genomic DNA digested with *Scal*, *BglII*, *XhoI*, *HincII* and *XbaI* respectively, lanes 7-9, rat genomic DNA digested with *HindIII*, *EcoRI* and *PstI* respectively. Bands between 2kb and 6kb shown in lanes with *Manduca* genomic DNA digested with *BglII*, *XhoI*, *HincII* and *XbaI*.

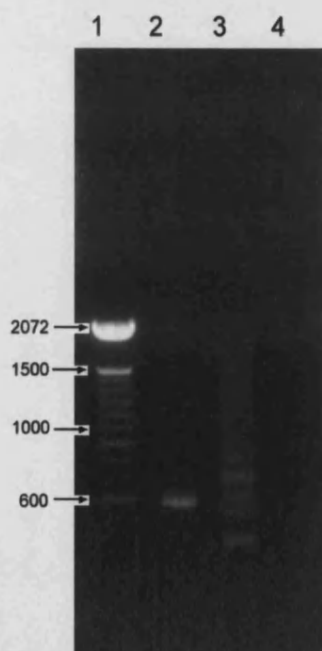


Figure 2.15. PCR on *HincII* digested and circularised genomic DNA from *Manduca*. Genomic DNA, PCR reaction mix (see section 2.3.4) and primers (see section 2.4.4.2) were incubated at 95°C for 5min, 2.5Units of *Taq* DNA Polymerase added and 35 cycles of 95°C for 1min, 55°C for 1min and 72°C for 1min were completed. Lane1, 100bp ladder (sizes in bp), lane 2, genomic DNA amplified with sense and antisense primers, lanes 3 and 4, negative controls, genomic DNA amplified with one primer only. A band of ~600bp shown in lane 2.

2.4.4.3 Sequencing analysis

The PCR product resulting from inverse PCR on *HincII* digested and circularised genomic DNA resulted in the following nucleotide and amino acid sequence (Figure 2.16). Analysis using the gcg program Fasta (Pearson and Lipman, 1988) showed identity with α -like subunits of the nAChR (Figure 2.17). Hereinafter, this sequence is called fragment a.

| | | |
|-----|--|---------------------|
| 1 | GTGCGAGGCCCAACGTGTGGAGTTGGGCGTCAACTCATGCGGAGTGTGACAAAATATTAC | 60 |
| -21 | | M R S V T K Y Y -14 |
| 61 | TTACATGGTGTGTTTGTGTTGCGACAGGATGTGCCGGCAACCCGACGCGAAGCGGCTG | 120 |
| -13 | L H G V V L F A T G C A G N P D A K R L | 7 |
| 121 | TACGACGATCTGCTCAGCAATTACAACAAGTTGGTGCGGCCTGTACTCAACGTCAGCGAC | 180 |
| 8 | Y D D L L S N Y N K L V R P V L N V S D | 27 |
| 181 | GCGCTCACCGTGCGCATCAAGCTCAAGCTGAGCCAGCTCATCGACGTGGTCAGCCGCCTT | 240 |
| 28 | A L T V R I K L K L S Q L I D V | 43 |
| 241 | <i>TAACACAAAAACGTCACAATCACTAGTTCTTACAATATCATACTGGAATCGTTGCAATAA</i> | 300 |
| 301 | <i>TAATAGCAATAGTTTAAACATTGTAATATAATTGTTGCAGAATCTCAAGAATCAAATAATG</i> | 360 |
| 44 | | N L K N Q I M 50 |
| 361 | ACAACCAATCTATGGGTGGAACAGGTTAGTCTCATTGTGTATGTGTTTGCACGATTATTG | 420 |
| 51 | T T N L W V E Q | 58 |
| 421 | <i>CAAGTTTGCATACAGTTTTTCTAGTAGCTATTGTGTTATTCGCAGAGCTGGTACGACTAC</i> | 480 |
| 59 | | S W Y D Y 63 |
| 481 | AAGCTTTCGTGGGAGCCGCGCGAGTACGGCGGCGTGGAAATGCTGCACGTGCCTTCCGAC | 540 |
| 64 | K L S W <u>E P R E Y G G V E M L H V P S D</u> | 83 |
| 541 | CACATCTGGCGGCCCGACATCGTGCTTTACAACAATGCTGAC | 582 |
| 84 | <u>H I W R P D I V L Y N N A D</u> | 97 |

Figure 2.16. Nucleotide and amino acid sequence of fragment a. Intronic sequences are shown in italics. Sequence which overlaps with fragment c is underlined.

1) *D. melanogaster* mRNA for nicotinic acetylcholine receptor alpha-like subunit (ALS)
 ACCESSION NUMBER X07194

64.0% identity in 150 bp overlap

| | | | | | | |
|--------|---|------|------|------|------|------|
| | 50 | 60 | 70 | 80 | 90 | 100 |
| frag a | ACAAAATATTACTTACATGGTGTGTTTGTGTTGCGACAGGATGTGCCGGCAACCCCGAC | | | | | |
| | | | | | | |
| ALS | CTATTTCGCAGCTGTATTTCATAGCATTACACTTTGCCACCGCGGCCTGGCCAACCCAGAT | | | | | |
| | 1300 | 1310 | 1320 | 1330 | 1340 | 1350 |

| | | | | | | |
|--------|--|------|------|------|------|------|
| | 110 | 120 | 130 | 140 | 150 | 160 |
| frag a | GCGAAGCGGCTGTACGACGATCTGCTCAGCAATTACAACAAGTTGGTGCGGCCTGTACTC | | | | | |
| | | | | | | |
| ALS | GCGAAGCGACTCTACGACGACCTGCTGAGCAACTACAATCGCCTCATCCGACCCGTGGGC | | | | | |
| | 1360 | 1370 | 1380 | 1390 | 1400 | 1410 |

| | | | | | | |
|--------|--|------|------|------|------|------|
| | 170 | 180 | 190 | 200 | 210 | 220 |
| frag a | AACGTCAGCGACGCGCTCACCGTGCGCATCAAGCTCAAGCTGAGCCAGCTCATCGACGTG | | | | | |
| | | | | | | |
| ALS | AACAACTCGGACCGTCTCACCGTCAAGATGGGTCTGCGCCTCTCCAGCTGATCGATGTG | | | | | |
| | 1420 | 1430 | 1440 | 1450 | 1460 | 1470 |

2) *S. gregaria* mRNA for neuronal nicotinic acetylcholine receptor (α L1/ARL2)
 ACCESSION NUMBER X55439

77.2% identity in 114 bp overlap

| | | | | | | |
|--------|---|-----|-----|-----|-----|-----|
| | 440 | 450 | 460 | 470 | 480 | 490 |
| frag a | TTCTAGTAGCTATTGTGTTATTTCGCAGAGCTGGTACGACTACAAGCTTTCGTGGGAGCCG | | | | | |
| | | | | | | |
| ARL2 | CTTACCACCAACGTCTGGTTGGAGCACGAATGGCAGGACCACAAGTTCCGATGGGACCCG | | | | | |
| | 240 | 250 | 260 | 270 | 280 | 290 |

| | | | | | | |
|--------|---|-----|-----|-----|-----|-----|
| | 500 | 510 | 520 | 530 | 540 | 550 |
| frag a | CGCGAGTACGGCGGCGTGGAAATGCTGCACGTGCCTTCCGACCACATCTGGCGGCCCCGAC | | | | | |
| | | | | | | |
| ARL2 | GCGGAGTACGGAGGCGTGACGGAGCTGTACGTCCCTTCGGAGCACATCTGGCTGCCCCGAC | | | | | |
| | 300 | 310 | 320 | 330 | 340 | 350 |

| | | | |
|--------|--|-----|-----|
| | 560 | 570 | 580 |
| frag a | ATCGTGCTTTACAACAATGCTGAC | | |
| | | | |
| ARL2 | ATCGTCCTGTACAACAACGCTGACGGCGAGTACGTGGTCACGACCATGACTAAGGCGGTG | | |
| | 360 | 370 | 380 |

Figure 2.17. Alignment of part of fragment a with part of the sequences encoding ALS from *Drosophila* and α L1 (ARL2) from *Schistocerca*. These two sequences represent the highest nucleotide identities obtained from searching the Genbank and EMBL databases using the gcg program Fasta (Pearson and Lipman, 1988).

Fragment a is 582bp in length. Of this, 90bp (30 amino acid residues) overlaps with fragment c and contained within it, is the entire sequence of fragment b. As the overlapping sequences are identical, it suggests that fragments a, b and c are from the

same α -like subunit, which appears to be found in both embryonic and pupal stages of the life cycle.

Fragment a represents the complete coding sequence missing from fragment c. Interestingly, it comprises 3 exons and 2 introns. The 3 exons are 192, 45 and 117bp in length and are separated by extremely short intronic sequences of 111 and 81bp. In each case, there appears to be a donor consensus sequence of GGTNAG and an acceptor consensus sequence of TT/CGCAGA where "N" represents any nucleotide. This is in agreement with invertebrate consensus sequences described by Senapathy *et al.* (1990). The positions of the introns correspond to the first two introns of ALS (Bossy *et al.*, 1988) and the first intron of D α 2/SAD (Jonas *et al.*, 1990) (see Figure 1.7). There are 36 nucleotides prior to the presumed initiation codon, which may represent 5' untranslated sequence when converted to cDNA. However, as there appears to be no sequence corresponding to a TATA box in fragment a, the transcription start site cannot be located. The presumed initial coding sequence represents a signal peptide sequence of 20-21 amino acid residues. Analysis using the program Sigseq, based on von Heijne's rules (1983, 1986), gives two alternatives for the signal peptide cleavage site. The one with the highest probability is the sequence CA/GNPD, where "/" represents the site of cleavage. Slightly less likely (but corresponding to those signal peptide cleavage sites predicted for other insect α -like subunits) is the sequence CAG/NPD. Accordingly, the nomenclature used in numbering amino acid residues in fragments a and b corresponds to that found in other insect α -like subunits, i.e. amino acid residue number 1 is the asparagine after the predicted signal peptide cleavage site CAG/NPD.

2.4.5 Attempts to obtain a full-length MARA1

2.4.5.1 Preliminary observations

For the synthesis of a *de novo* copy of the full-length MARA1 or the construction of a hybrid from the fragments obtained, it was necessary to confirm that fragments a, b and c were all part of the same α -like subunit, i.e. MARA1. Given that there is 100% nucleotide and amino acid identity in the overlapping regions between fragment a, b and c, it is highly likely that the three fragments represent DNA encoding the same α -like receptor.

Southern blot analysis

The palindromic sequences of restriction enzymes known as rare cutters arise so infrequently, that they could, in theory, result in the entire genomic clone of MARA1, which all the fragments would recognise.

Therefore, genomic DNA was digested with the restriction enzymes *NotI*, *BglI*, *EcoRI* and *SacI*, the samples precipitated and run on a 0.6% (w/v) agarose gel and transferred to nylon membrane by Southern blotting, as described in section 2.3.5. Fragments a, b and c were excised from pBluescript at appropriate restriction sites, purified using GeneClean® II and used sequentially as radiolabelled probes for hybridisation to the Southern blot, using techniques described in section 2.3.5.

As seen in Figure 2.18, parts a, b and c, the pattern of bands observed for each autoradiograph, corresponding to the digested genomic DNA probed with each radiolabelled fragment was almost identical. Comparison with the original photograph of the gel (not shown) gave the following identical bands for each autoradiograph: For DNA digested with *BglI*, bands were evident at approximately 10kb and 8 kb; for DNA digested with *EcoRI*, bands appeared at approximately 12kb and 10kb and for DNA digested with *SacI* there was one band between 6 and 7kb. Fragment b and c probes also resulted in a band at approximately 3kb for DNA digested with *EcoRI*. Furthermore, a band of size greater than 12kb was observed for DNA digested with *BglI* and probed with fragment b.

The genomic DNA could accommodate a number of nAChR α -like subunits and/or β -like subunits, thus differences in the pattern of bands arising on the autoradiographs would be dependent on the degree of homology between these hypothetical α -like or β -like subunits and MARA1. Other possible explanations for different band patterning include intronic restriction enzyme sites, by their very nature not present in the cDNA, and alternative splicing of the gene encoding the nAChR.

Overall, the pattern of bands for each fragment is so similar that it makes it unlikely that the fragments are derived from different α -like subunits. Comparisons of the sequence data from each fragment shows that the overlapping amino acid and more importantly, nucleotide sequences are identical. As fragments a and b overlap fragment c by 90bp and as fragment b is entirely contained within fragment a, the two anomalous bands may be due to experimental error. This spurious result may not be gene specific hybridisation - possibly the radioactivity is more concentrated in the region of fragment b than fragment a.

a)

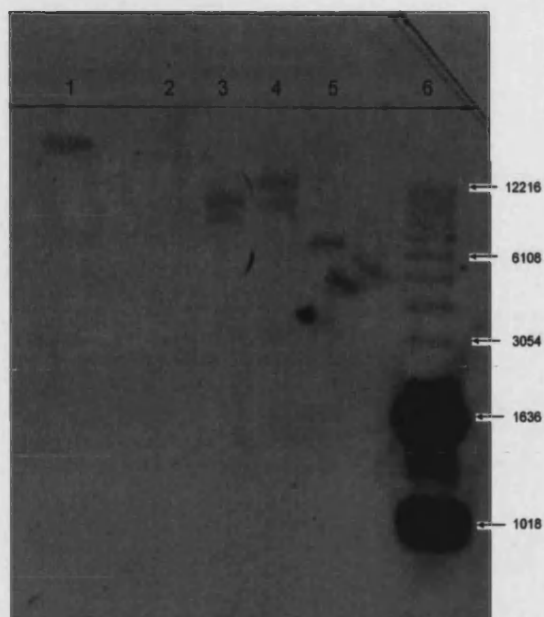
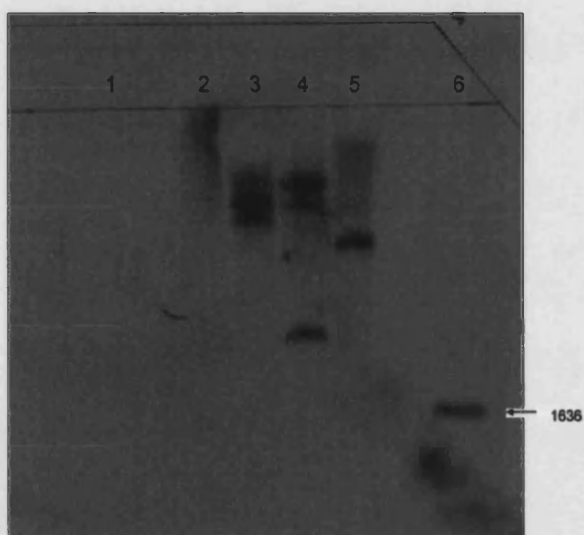
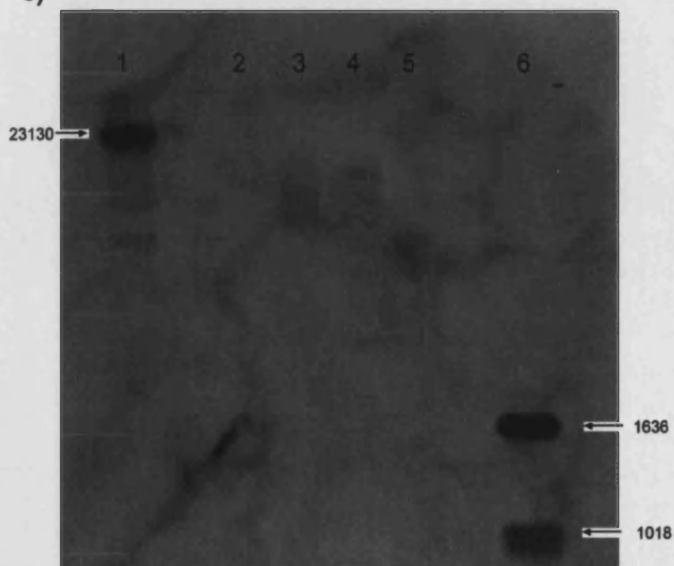


Figure 2.18. Autoradiographs from the hybridisation of Southern blot of digested genomic DNA with each fragment encoding the α -like subunit of the nAChR in *Manduca*. a) blot probed with fragment a; b) blot probed with fragment b and c) blot probed with fragment c. See text for experimental details. Lane 1, λ DNA digested with *Hind*III (sizes in bp); lane 2, DNA digested with *Not*I; lane 3, DNA digested with *Bgl*II; lane 4, DNA digested with *Eco*RI; lane 5, DNA digested with *Sac*I; lane 6, 1kb ladder (sizes in bp). Similar banding patterns are shown for each fragment.

b)



c)



PCR analysis

PCR was used to splice by overlap extension (Horton *et al.*, 1993) fragments a and c, to show that the overlapping sequence was real. PCR on fragments a and c used the primer sense 6 to fragment a (sequence: 5' AAGAATCAAATAATGACAACC 3' at nucleotide positions 346-366 of fragment a; see Appendix 2, parts b and c), upstream of the overlapping region and the primer antisense 1 to fragment c (sequence: 5' GGACAGCGATACCTTCTC 3' at nucleotide positions 544-561 of fragment c; see Appendix 2, parts a and c), downstream of the overlapping region. PCR resulted in a product, shown in Figure 2.19, of approximately 700bp (size expected 708bp).

Direct sequencing of the PCR product using an automated Perkin Elmer ABI PRISM™ 377 DNA Sequencer gave the following sequence, Figure 2.20, hereinafter called fragment d.

| | | |
|-----|---|-----|
| 1 | TGGGTGGAACAGGTTAGTCTCATTGTGTATGTGTTTGCACGATTATTGCAAGTTTGCATA | 60 |
| 55 | W V E Q | 58 |
| 61 | CAGTTTTTCTAGTAGCTATTGTGTTATTCGCAGAGCTGGTACGACTACAAGCTTTCGTGG | 120 |
| 59 | S W Y D Y K L S W | 67 |
| 121 | GAGCCGCGCGAGTACGGCGGCGTGAAATGCTGCACGTGCCTTCCGACCACATCTGGCGG | 180 |
| 68 | E P R E Y G G V E M L H V P S D H I W R | 87 |
| 181 | CCCGATATCGTGCTTTACAACAATGCTGACGGCAACTTTGAGGTGACGCTGGCGACTAAG | 240 |
| 88 | P D I V L Y N N A D G N F E V T L A T K | 107 |
| 241 | GCGACGCTCAATTACACGGCCGAGTGGAGTGGCGTCCGCCCGCCATCTACAAGTCCTCG | 300 |
| 108 | A T L N Y T G R V E W R P P A I Y K S S | 127 |
| 301 | TGCGAGATCGACGTAGAGTACTTTCCATTTCGACCAGCAGACGTGCGTCATGAAGTTTGGC | 360 |
| 128 | C E I D V E Y F P F D Q Q T C V M K F G | 147 |
| 361 | TCGTGGACCTACGACGGGTTTCAGGTAGATCTGCGACATATCGATGAAGTGCAGGCACC | 420 |
| 148 | S W T Y D G F Q V D L R H I D E V R G T | 167 |
| 421 | AACGTCGTGGAGTTGGGCGTCGACCTCTCCGAGTTTTACACCTCTGTCGAGTGGGACATC | 480 |
| 168 | N V V E L G V D L S E F Y T S V E W D I | 187 |
| 481 | CTGGAGGTGCCCCCGTCCGAAATGAAAAGTTCTACACGTGCTGCGACGAGCCCTATCTA | 540 |
| 188 | L E V P A V R N E K F Y T C C D E P Y L | 207 |
| 541 | GATATAACTTTCAACATCACGATGCGCCGAAAACACTCTTCTACACGTTAATCTGATC | 600 |
| 208 | D I T F N I T M R R K T L F Y T V N L I | 227 |
| 601 | ATTCCGTGCATGGGAATATCGTTTCTGACG | 630 |
| 228 | I P C M G I S F L T | 237 |

Figure 2.20. Nucleotide and amino acid sequence of fragment d. Amino acids that are underlined represent overlap between fragments a and c.

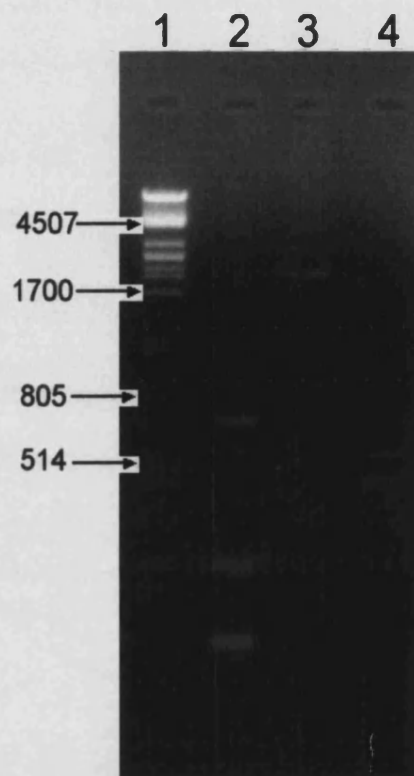


Figure 2.19. PCR to splice together fragments a and c. DNA, PCR reaction mix (see section 2.3.4) and primers (see section 2.4.5.1) were incubated at 95°C for 5min, 2.5Units of *Taq* DNA Polymerase was added and 40 cycles of 95°C for 1min, 37°C for 1min and 72°C for 1min were completed. Lane 1, λ DNA digested with *Pst*I (sizes in bp); lane 2, fragments a and c amplified with both primers; lanes 3 and 4, negative controls, fragments a and c amplified with one primer only. A band of ~700bp shown in lane 2.

Fragment d represents the overlapping sequence of fragments a and c, in addition to the sequences directly downstream and upstream of the sense and antisense primers respectively. Fragment d is 630bp in length, 90bp of which corresponds to the overlapping sequence, 120bp of which corresponds to solely fragment a sequence and 420bp of which corresponds to solely fragment c sequence.

As fragments from different subunits would not be expected to anneal to form a stable product, this hybrid of fragments a and c, produced by PCR, implies that fragments a and c are of the same subunit gene.

2.4.5.2 Searching for the full-length clone

An embryonic developmental profile

In order to facilitate the finding of a full-length clone, a preliminary study on the developmental profile of the α -subunit of the nAChR in *Manduca* eggs was carried out. In theory, a semi-quantitative PCR to amplify a short fragment of the α -subunit should indicate the day at which the amount of nAChR mRNAs are at their highest, thus giving the greatest chance of obtaining a full-length clone.

Total RNA and poly [A⁺] RNA were isolated from day 0, 1, 2, 3, and 4 *Manduca* eggs as described in section 2.3.1. First strand cDNA was synthesised as previously but using a ramped reverse transcription protocol of 37°C for 30min, 42°C for 30min and 50°C for 30min, as it was thought that elevated temperatures may alleviate problems caused by any secondary structure in the mRNA and thus result in full-length cDNA rather than truncated versions. Analysis of radiolabelled first strand cDNA on an alkaline agarose gel and subsequent autoradiography revealed cDNA up to 4kb in length. Estimation by eye suggested less synthesis of the cDNA from day 0 mRNA compared with days 1-4 (data not shown).

Nested PCR on the first strand cDNA was performed using an outer pair (sequences: sense 7, 5' CTCGATACGTTTCAGCATATGCGTG 3' at nucleotide positions 673-696 of fragment c, see Appendix 2, parts a and c; antisense 5, 5' GGCCGTGTACGCTATCTCCGACAG 3' at nucleotide positions 1334-1357 of fragment c, see Appendix 2, parts a and c) and inner pair (sequences: sense 8, 5' AGTCGCTTCGCAGGACTGGTGACC 3' at nucleotide positions 835-858 of fragment c, see Appendix 2, parts a and c; antisense 6, 5' CAGGAATGGTCTGTCGAGTACCAT 3' at nucleotide positions 1117-1140 of fragment c, see Appendix 2, parts a and c) of primers to the C-terminal region of MARA1. A first round PCR with the outer pair of primers

resulted in a PCR product of approximately 600bp (expected size 591bp) for the positive control of fragment c only (Figure 2.21). A second round hotstart PCR with the inner pair of primers and a 1 in 100 dilution of the first round product, using the same cycling conditions resulted in a PCR product of approximately 300bp (expected size 347bp) for both the positive control of fragment c and for first strand cDNA from each day. Visual analysis indicated that the quantity of PCR product made with day 4 eggs was far less than that for days 0-3, which showed a similar level of product (Figure 2.21). This finding was confirmed through Southern blot analysis, using a probe constructed from the C-terminal sequence of fragment c (Figure 2.22). Consequently first strand cDNA from days 2 and 3 was used to search for the full-length MARA1.

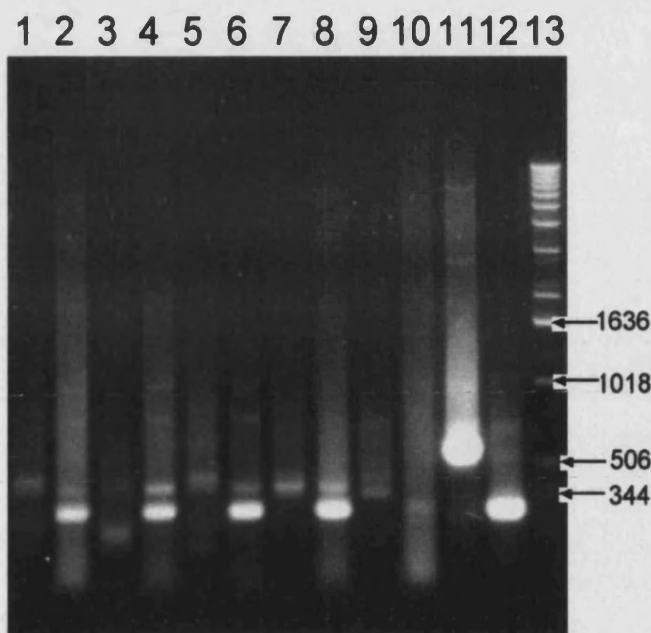


Figure 2.21. Nested PCR on first strand cDNA. The first round of amplification (1°) used 40 cycles of 95°C for 1min, 50°C for 1min and 72°C for 1min, after the initial 5min at 95°C and addition of *Taq* DNA Polymerase to the cDNA, PCR reaction mix and primers. The second round of amplification (2°) used a 1 in 100 dilution of the first round product, and the same cycling conditions. PCR products were analysed on 1% (w/v) agarose gel. Lanes 1 and 2; day 0, lanes 3 and 4, day 1; lanes 5 and 6, day 2; lanes 7 and 8, day 3; lanes 9 and 10, day 4; lanes 11 and 12, positive control using fragment c. In each doublet of lanes, the first of that day represents the 1° round and the second represents the 2° round. Lane 13, λ DNA digested with *Hind*III (sizes in bp). Bands of ~300 bp shown for each 2° round.

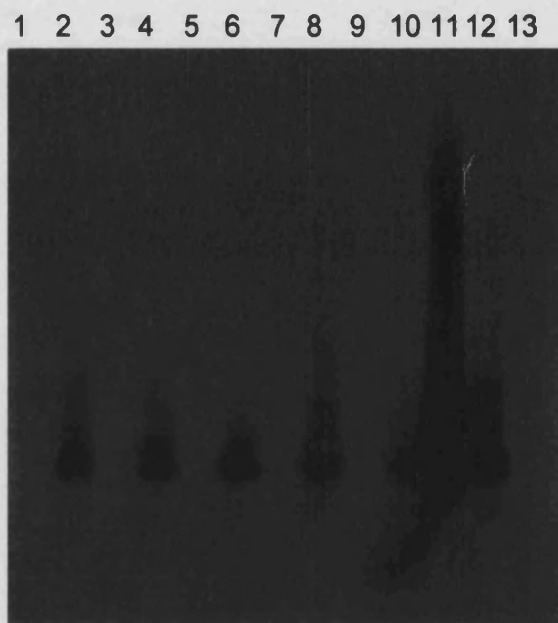


Figure 2.22. Autoradiograph of Southern blot of nested PCR on first strand cDNA. PCR products in gel were transferred to nylon membrane by Southern blotting and the blot hybridised with a probe constructed from fragment c, as described in section 2.3.5. Lanes 1 and 2, day 0; lanes 3 and 4, day 1; lanes 5 and 6, day 2; lanes 7 and 8, day 3; lanes 9 and 10, day 4; lanes 11 and 12, positive control using fragment c. In each doublet of lanes, the first of that day represents the 1° round and the second represents the 2° round. Lane 13, λ DNA digested with *Hind*III. Bands of ~300 bp shown for each 2° round (by measurement from analytical gel, Figure 2.21).

Searching for a de novo copy

Specific primers to the N-terminal end of fragment a and the C-terminal end of fragment c, incorporating different and non-compatible restriction enzyme sites, were used in PCR reactions with the proofreading Vent_R® DNA Polymerase (Mattila *et al.*, 1991). These were the primers, sense 9 and 10 incorporating a *Bam*HI site (sequences: 5' CGCGGATCCGTGCGAGGCCCAACGTGTG 3' at nucleotide positions 1-19 of fragment a, see Appendix 2, parts b and c; 5' CGCGGATCCATGCGGAGTGTGACA 3' at nucleotide positions 37-51 of fragment a, see Appendix 2, parts a and c; respectively) and antisense primer 7 incorporating either an *Ap*al or *X*hoI site (sequences: 5' GCACGGGCCCATACAAACGATCATTTTATTTTCGTC 3'; 5' CCCGCTCGAGATACAAACGATCATTTTATTTTCGTC 3' both at nucleotide positions 1600-1624 of fragment c and only differing in restriction site, see Appendix 2, parts a and c respectively). Standard PCR conditions, increasing the annealing temperature, using a second round - effectively doubling the number of cycles, and including formamide in the reaction mixture (Sarkar *et al.*, 1990), all failed to produce a full-length clone. The smears present on the analytical gel shown in Figure 2.23 suggested a lack of template cDNA of the appropriate size.

In addition, Expand™ (Boehringer Mannheim) - a long template DNA Polymerase system and GeneAmp® (Perkin Elmer) - a thermostable Reverse Transcriptase that operates at 70°C and also functions as a DNA Polymerase for PCR (Myers and Gelfand, 1991) were used for reverse transcription and PCR according to the manufacturer's protocols. Neither were successful in producing a full-length clone of MARA1.

The lack of a full-length clone from cDNA suggests that in each case the reverse transcript for MARA1 was truncated. In addition, an excessive number of cycles was necessary to isolate an internal fragment of MARA1. Generally, 1µg of poly [A+] RNA contains ~5x10⁷ copies of each low abundance transcript. PCR works well with as little as 10³ templates in the starting mixture suggesting that the mRNA for MARA1 is in very low abundance (Frohman, 1993).

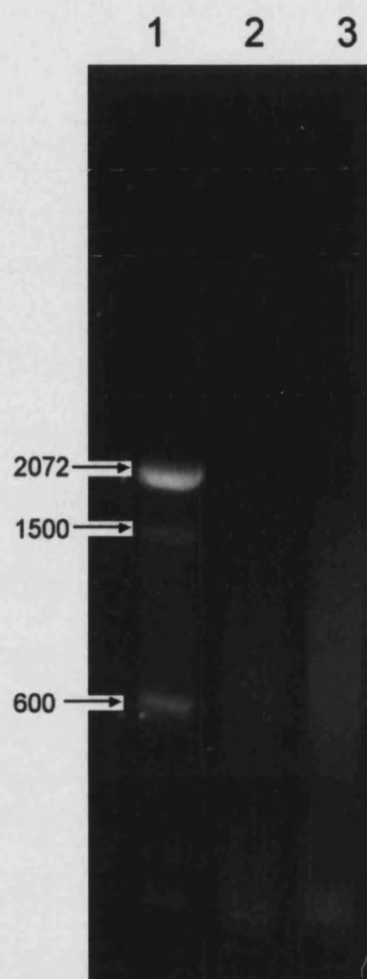


Figure 2.23. PCR on first strand cDNA using primers specific to the extreme ends of sequence encoding the MARA1 subunit. cDNA, PCR reaction mix (see section 2.3.4) and primers (see text: Searching for a *de novo* copy) were incubated at 96°C for 5min, 2.5Units of Vent DNA Polymerase was added and 40 cycles of 95°C for 1min, 45°C for 1min and 72°C for 2min were completed. PCR products were analysed on an agarose gel. Lane 1, 100bp ladder (sizes in bp); lane 2 and 3, representative smears, suggesting lack of template DNA from days 2 and 3 cDNA respectively using primers sense 9 and antisense 7.

Ligation of fragments a and c

The ligation of fragments a and c was also attempted. Following the successful isolation of fragment d using splicing by overlap extension, the same technique was applied to the whole fragments (Figure 2.24), using the specific primers to the N-terminal end of fragment a and the C-terminal end of fragment c described above.

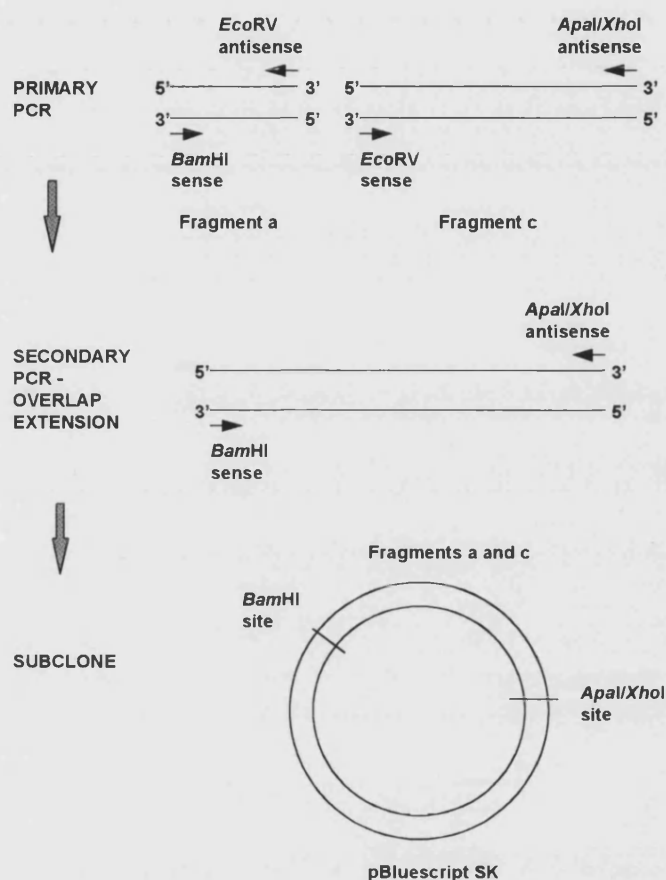


Figure 2.24. Splicing by the overlap extension PCR method.

(Not to scale).

Primary PCR: Fragment a amplified with sense 9 (nucleotide positions 1-19 of fragment a) and antisense 8 (nucleotide positions 55-75 of fragment c), incorporating *Bam*HI and *Eco*RV sites respectively. Fragment c amplified with sense 11 (nucleotide positions 55-75 of fragment c) and antisense 7 (nucleotide positions 1600-1624 of fragment c), incorporating *Eco*RV and either *Apal* or *Xho*I respectively (see Appendix 2).

Secondary PCR: Fragments a and c spliced together using sense 9 and antisense 7.

Resulting PCR product subcloned into pBluescript using restriction sites at either end of PCR product.

Despite alterations in the cycling conditions, the full-length clone was not amplified. Given that this technique produces a shorter overlapping fragment, (fragment d, Figure 2.20) it seems strange that a longer overlapping fragment cannot be produced. This may be due to the disparities in size difference between the two fragments. Fragment c is approximately 3x larger than fragment a and this may create an unstable hybrid which is not readily amplified to produce a full-length fragment but is amplified for a shorter, more stable product.

The megaprimer technique, Figure 2.25 was also used. Here, fragment c is amplified by a sense megaprimer, namely fragment a, and antisense primer 7, specific to the C-terminal of fragment c. In theory, this should result in a PCR product of fragment a

overlapping fragment c, however, this was not the case. Again, this may be due to the disparities in size difference between the two fragments.

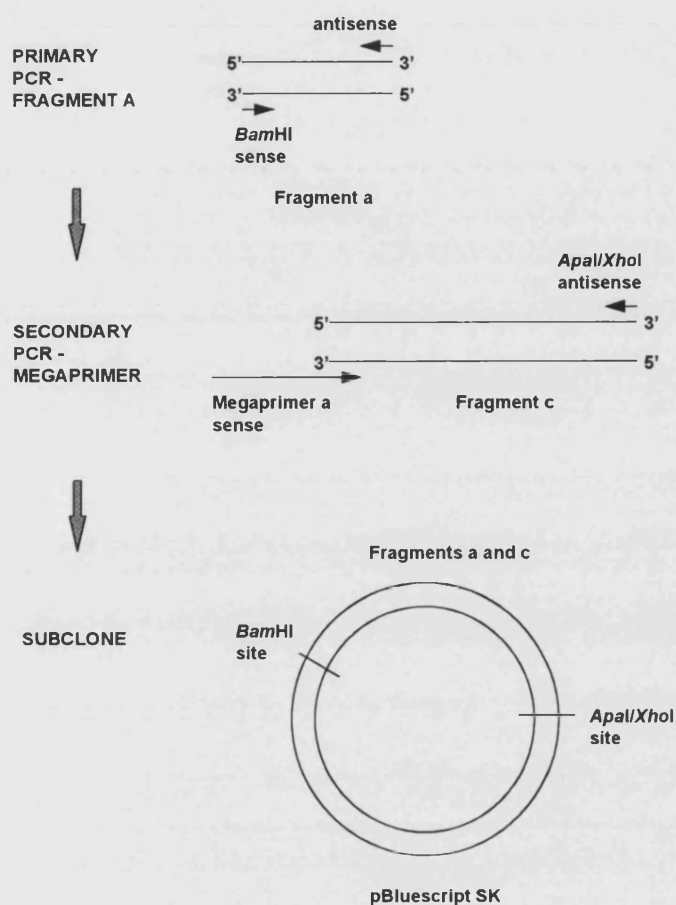


Figure 2.25. Splicing by the megaprimer PCR method.

(Not to scale)

Primary PCR: Fragment a amplified with sense 9 (nucleotide positions 1-19 of fragment a) and antisense 8 (nucleotide positions 55-75 of fragment c).

Secondary PCR: Fragment c amplified with fragment a (the megaprimer) and antisense 7 (nucleotide positions 1600-1624 of fragment c).

Resulting PCR product subcloned into pBluescript using restriction sites at either end of PCR product.

A final attempt to splice the two fragments together by engineering a mismatch *Eco*RV restriction site into the C-terminal end of fragment a and the N-terminal end of fragment c (Figure 2.26) (sense 11, sequence: 5' TGGCGGCCCGATATCGTGCTT 3'; antisense 8, sequence: 5' AAGCACGATATCGGGCCGCCA 3' at nucleotide positions 55-75 of fragment c, i.e. within overlap of fragments a and c, see Appendix 2, parts a and c; note incorporation of *Eco*RV restriction site into primers, i.e. MARA1 sequence, starting at nucleotide 64 of fragment c is GACAT whereas primer is GATAT) was partially successful.

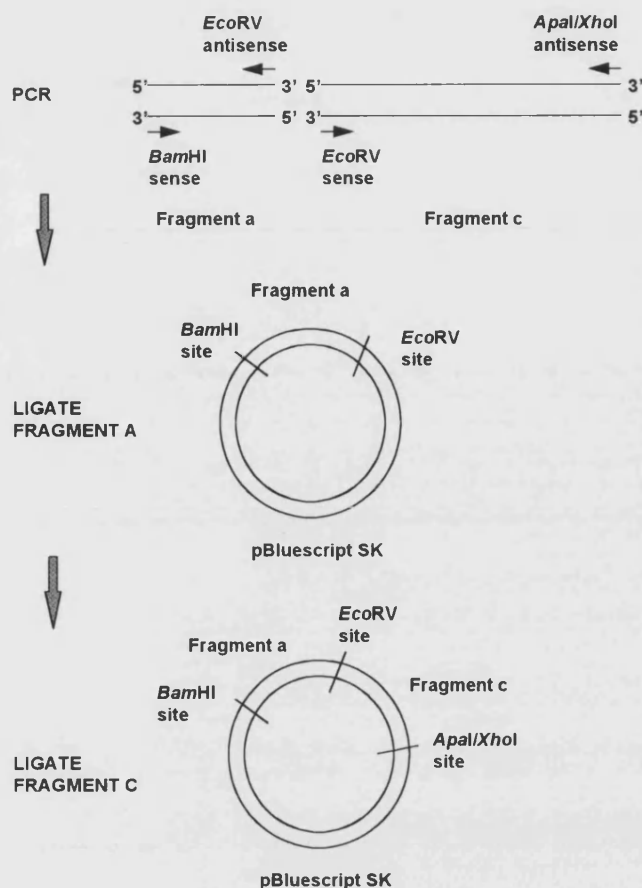


Figure 2.26. Splicing by engineered restriction sites.

(Not to scale).

Fragment a amplified with sense 9 and antisense 8. Fragment c amplified with sense 11 and antisense 7 (see text for primer details).

Fragment a ligated into pBluescript using restriction sites at either end of PCR product. Fragment c ligated into the fragment a containing pBluescript using restriction sites at either end of PCR product, the N-terminal one corresponding to that of the C-terminal of fragment a.

Fragment a was amplified with a *Bam*HI site at the N-terminal end (as above) and with a mismatch *Eco*RV site at the C-terminal end by PCR and subcloned into pBluescript at the corresponding restriction sites. Fragment c was amplified with a mismatch *Eco*RV site at the N-terminal end and either an *Apal* or *Xho*I site at the C-terminal end (as above) and subcloned into pBluescript containing fragment a at the corresponding restriction sites. This technique however resulted in a fragment that was composed of fragment a ligated to only part of fragment c, thus giving another partial length fragment but not the full-length clone.

Searching for a genomic clone

Given the lack of a full-length clone from cDNA, attempts to isolate the genomic clone were made. This utilised PCR with the long template DNA polymerase system called Expand™ (see section 2.3.4). As the size of the genomic clone could not be estimated, three buffers supplied with the enzyme, giving various reaction conditions were used. The primers sense 9 incorporating a *Bam*HI restriction site (sequence 5' CGCGGATCCGTGCGAGGCCCAACGTGTG 3' at nucleotide positions 1-19 of fragment a) and antisense 7 incorporating an *Apal* restriction site (sequence 5'

GCACGGGCCCCATACAAACGATCATTTTATTTTCGTC 3' at nucleotide positions 1600-1624 of fragment c) were used. Controls using single primers were also carried out.

Expand™ PCR resulted in a doublet of PCR products, the upper band approximately 6kb and the lower band approximately 5kb (Figure 2.27) which were not observed in the single primer controls. The PCR product was purified and approximately the first 700bp were sequenced on an automated Perkin Elmer ABI PRISM™ 377 DNA Sequencer. The sequence is shown in Figure 2.28. Sequencing ambiguities and analysis of the sequence using the gcg program Fasta (Pearson and Lipman, 1988), suggested that the purified product was not an α -like subunit of the nAChR (Figure 2.29).

| | | |
|-----|--|-----|
| 1 | TTTTGTGCGAGGcagGCGCCGTTTCaGAAaTACGACCTTTCGGGCCGAGg | 50 |
| 51 | GAACTAgCAAACGGCTTCCCCaATTTTCCATCTaATGaAaAtgCGGtcGAG | 100 |
| 101 | TATCAcAcCTgGGTcTAtCCTTGTCgTCcCTACATCATCTaCAcATGT | 150 |
| 151 | tCCCATCACGTTAccGTATcTcCCTCtAAATTCACGTCTGGTCGTACTCC | 200 |
| 201 | GCcTAGGGaGTGGcCCGATATcTGGAgAAATAAAACAtCtGGgAAAaCAG | 250 |
| 251 | TGAaTATGTTCTTcAtAACcaCGagTTTATtCGGtACTTTCGGTcCcCGT | 300 |
| 301 | GaacGTggcATAATCGTCCGGTcTATTggGATTCTaTAcGACaTtCCCCG | 350 |
| 351 | CNgGctATTGAcgGGACATGATGAcCCCgCCCCgTTCAGTTTGTgaCcAt | 400 |
| 401 | ATccCATCCCCCGgCCCCgGaCcTATATcGATGTCTGATCTGGGCCtacC | 450 |
| 451 | GTTGCCCCCTCAAaGTGTcATtCTATCCACcGcGtGaGCgATCCGTTTTTCg | 500 |
| 501 | TTCcATaGAAtcACCCCGGTGCCCTCTAAAtcAAATgTCTTgCCCAGAAAt | 550 |
| 551 | gGAtACTTgTAcATCCgtCGCAAGCTCCTgTTACTGgGGTgTTTaTTgta | 600 |
| 601 | CCcTCCCCCGTGTGTcGGAtATaTTAACTGCgCCcCNNGGTtATTTTTTA | 650 |
| 651 | NTTCCNGGACNANTANNNNACTNAANAANCCGNGGTNTGAAATNNCCTC | 700 |
| 701 | NGGGNANTCCCNCTTCNGACT | 722 |

Figure 2.28. Partial nucleotide sequence of the genomic PCR product. Lower case letters represent unclear sequence while the letter N, represents any nucleotide.

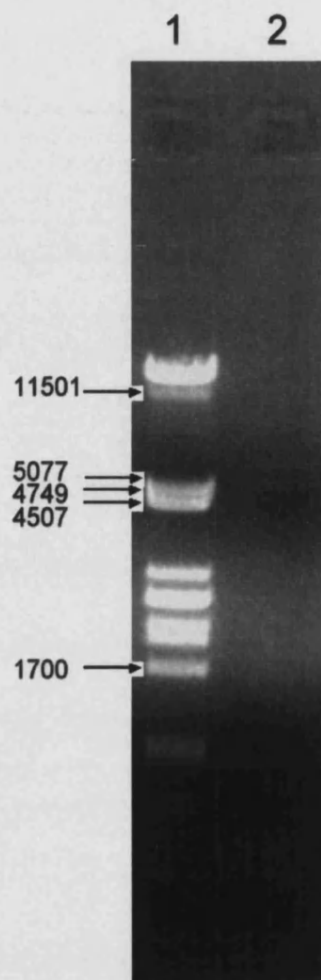


Figure 2.27. PCR on genomic DNA using primers specific to extreme ends of the sequence encoding MARA1. Genomic DNA, PCR reaction mix containing buffer 1 (see section 2.3.4) and Expand™ long template DNA Polymerase were incubated at 94°C for 2min and then subjected to 10 cycles of 94°C for 10s, 55°C for 30s and 68°C for 12min, followed by 20 cycles of the same conditions but with an extra 20s for each extension step of each cycle. Any PCR products were subjected to a final extension of 68°C for 7min. Lane 1, λ DNA digested with *Pst*I (sizes in bp); lane 2, genomic DNA amplified with sense 9 and antisense 7, showing a doublet of PCR products at ~5000bp.

1) *H.sapiens* DMA, DMB, HLA-Z1, IPP2, LMP2, TAP1, LMP7, TAP2, DOB, DQB2
 ACCESSION NUMBER X87344

61.3% identity in 106 bp overlap

```

      130      140      150      160      170      179
gen.se  TTGTACGTCCTACATCATCTACACATGTTCCCATCACGTTACCGTATCT--CCCTCTAA
      | | | | | | | | | | | | | | | | | | | | | | | | | | |
hsevmh  AAAGAAGGGGACACACGCCTCTTCCACCCGCTCATGCTGTTACTGCATCTGATCATCTCC
      134000      134010      134020      134030      134040      134050

      180      190      200      210      220      230
gen.se  ATTCACGTCTGGTTCGT--ACTCCGCCT-----AGGGAGTGGCCCGATATCTGGA-GAAA
      | | | | | | | | | | | | | | | | | | | | | | | | | | |
hsevmh  GTGC-CGTGTCCTTGTTCACTTAGCCTGTGTTGAGTGTGTTGTCTCCATTTCCAAATGCAA
      134060      134070      134080      134090      134100      134110

      240      250      260      270      280      290
gen.se  TAAAACATCTGGGAAAACAGTGAATATGTTCTTCATAACCACGAGTTTATTCGGTACTTT
      | | | | | | | | | | | | | | | | | | | | | | | | | | |
hsevmh  TAAAACATCTGGGAAAGACTAAGGTAGGTGTGGGCAGGAAGAAGGGAGGAAGTTAGACCC
      134120      134130      134140      134150      134160      134170

```

2) *Marchantia polymorpha* mitochondrion, complete genome
 ACCESSION NUMBER M68929

68.2% identity in 44 bp overlap

```

      99      89      79      69      59      50
gen.se  TGTGATACTCGACCGCATTTTTCATTAGATGGAAAATGGGGAA--GCCGTTTGCTAGTTCC
      | | | | | | | | | | | | | | | | | | | | | | | | | | |
mpomtc  ATGTGACAGATAGATGGATATATAGAAAAAGAAAATGAGCGACCGGGGAGGCTAGTTCC
      42370      42380      42390      42400      42410      42420

      49      39      29      19      9
gen.se  CTCGGCCCCGAAAGGTCGTATTTCTGAAACGGCGCCTGCCTCGCACAAAA
      | | | | | | | | | | | | | | | | | | | | | | | | | | |
mpomtc  CGGGGACCGAAATGCGCAACGGTGACGAATTATCGCAAATGTGACGGAGATAGCAGGAAA
      42430      42440      42450      42460      42470      42480

```

3) *D. melanogaster* bithorax complex (BX-C), complete sequence
 ACCESSION NUMBER U31961

63.3% identity in 90 bp overlap

```

      100      110      120      130      140      150
gen.se  GCGGTCGAGTATCACACCTGGGTCTATTCTTGTACG-TCCCTACATCATCTACACATGT
      | | | | | | | | | | | | | | | | | | | | | | | | | | |
dm3196  CCTCATTATCGCCATATCTTGTGGCACAATTTTAACGATCCCAAATTA---ACACATGT
      117030      117040      117050      117060      117070      117080

      160      170      180      190      200      210
gen.se  TCCCATCACGTTACCGTATCTCCCTCTAAATTCACGTCTGGTCGTACTCCGCCTAGGGAG
      | | | | | | | | | | | | | | | | | | | | | | | | | | |
dm3196  TCCGCTAACGTTAACAGATTTTCAGGC--AATACCCCT---TC-TCCTCCCTCTGGGGAG
      117090      117100      117110      117120      117130

      220      230      240      250      260      270
gen.se  TGGCCCCGATATCTGGAGAAATAAACATCTGGGAAAACAGTGAATATGTTCTTCATAACC
      | | | | | | | | | | | | | | | | | | | | | | | | | | |
dm3196  CTCCATCGGGAAATTTATGAAGCGGTTTCGGTACGTTTCTGTCCCGCCTAAAATGCATTG
      117140      117150      117160      117170      117180      117190

```

Figure 2.29. Alignment of part of genomic DNA PCR product with non-nAChR sequences. Sequences shown represent the three highest nucleotide identities obtained from searching the Genbank and EMBL databases using the gcg program Fasta (Pearson and Lipman, 1988).

2.4.6 Searching for other α -like subunits

The presence of 3 α -like subunits of the nAChR in *Drosophila* (ALS, D α 2/SAD and D α 3, Bossy *et al.*, 1988; Baumann *et al.*, 1990, Jonas *et al.*, 1990, Sawruk *et al.*, 1990a; unpublished observations cited in Gundelfinger, 1992, respectively) suggests the likelihood of more than one α -like subunit in *Manduca*. Consequently, degenerate primers based on those used by Sgard *et al.* (1993) were used in PCR on first strand cDNA in an attempt to isolate partial length fragments of other α -like subunits.

Sequences of the degenerate primers were sense: 5' TACTAC/TA/CC/TCA/TC/GC/TGTC/GGAA/GTGGGACAT 3' and antisense: 5' GAAC/GAGC/TAGA/GTAC/TTTG/TCCC/GAGC/TAGCGG 3' giving degeneracy of 256-fold and 128-fold respectively. The positions of these primers are indicated on fragment c of Appendix 2, although the sequences of ALS, D α 2/SAD and α L1 were used to design the primers.

A positive control using fragment c resulted in a PCR product of approximately 300bp, close to the expected size of 332bp. However, not until the second round of conditions did PCR products for the first strand cDNA experimentals, corresponding to the same size seen in the positive control become visible when viewed on an agarose gel (figure 2.30). The PCR products were transferred to nylon membrane by Southern blotting and hybridised with radiolabelled fragment c as described in section 2.3.5. The autoradiograph obtained, Figure 2.31, showed hybridisation to the positive control from both rounds of PCR. Bands corresponding to PCR products from the second round of PCR, for day 2 and 3 eggs were observed. However, the band produced from hybridisation of the probe to day 2 cDNA was much fainter than the band for day 3. Furthermore, both bands appeared with far less intensity than the positive controls. The intensity of hybridised label observed on the autoradiograph does not correlate with the intensity of the bands seen on the agarose gel, Figure 2.30. If there was only one α -like subunit in the first strand cDNA corresponding to MARA1, i.e. the same sequence as fragment c, bands of similar intensities would be expected. Therefore, this anomaly suggests that there is more than one α -like subunit present in *Manduca*, resulting in bands of similar size with these degenerate primers. This is in keeping with the results observed with Sgard *et al.* (1993) who have suggested the presence of both ALS-like and D α 2/SAD-like subunits for *Myzus persicae*, as PCR results in one band that on sequencing, appears to consist of two subunits with degenerate primers at the same locations as those used in this study.

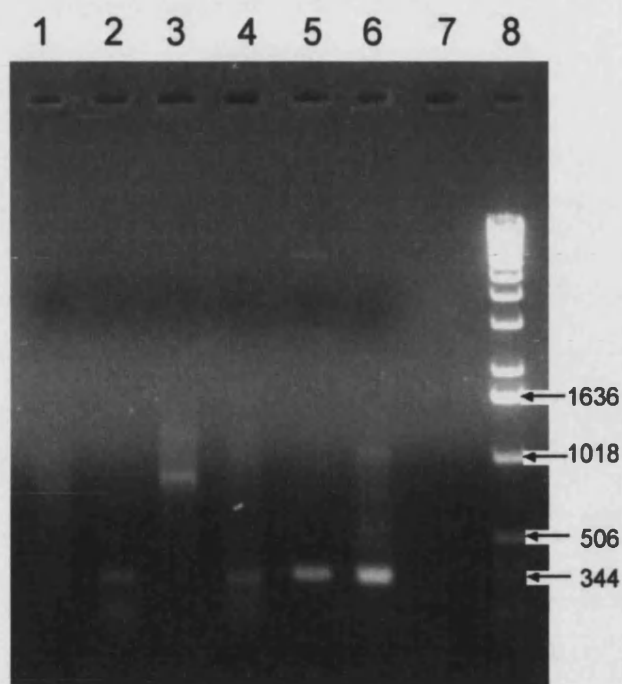


Figure 2.30. Amplification of further putative α -like subunits of the nAChR by PCR on first strand cDNA. PCR used first strand cDNA from days 2 and 3 *Manduca* eggs. cDNA, PCR reaction mix and primers were incubated at 96°C for 5min, 2.5Units of *Taq* DNA Polymerase was added and 40 cycles 95°C for 1min, 40°C for 1min and 72°C for 2min were completed. The PCR products from each day were diluted (1 in 50) and secondary rounds of PCR with the same cycling conditions except for an increased annealing temperature of 48°C were completed. PCR products were analysed on an agarose gel. Lanes 1 and 2, amplification of cDNA from day 2; lanes 3 and 4, amplification of cDNA from day 3; lanes 5 and 6, positive control, amplification with fragment c. For each doublet, the first lane represents the primary round and the second lane represents the secondary round. Lane 7, blank lane; lane 8, λ DNA digested with *Hind*III (sizes in bp). Bands of ~300bp shown in lanes 2, 4, 5 and 6.



Figure 2.31. Autoradiograph of Southern blot of PCR with degenerate primers on cDNA. PCR products in gel were transferred to nylon membrane by Southern blotting and the blot hybridised with a probe constructed from fragment c, as described in section 2.3.5. Lanes 1 and 2, amplification of cDNA from day 2; lanes 3 and 4, amplification of cDNA from day 3; lanes 5 and 6, positive control, amplification with fragment c. For each doublet, the first lane represents the primary round and the second lane represents the secondary round. Bands corresponding to PCR products from the second round of PCR, for day 2 and 3 eggs shown, in addition to bands from the positive control, fragment c.

Attempts to sequence the PCR products resulted in ambiguous sequence data (Figure 2.32) that did not correlate well with any other sequences in the Genbank and EMBL databases (Figure 2.33). As these degenerate primers were designed to sequences upstream of the internal primers used in the developmental study, it is possible that the cDNA strands were truncated, resulting in the amplification of non-specific products.

| | | |
|-----|--|-----|
| 1 | GAACAGCAGATATTTTCCAGTAGCGGANACAATAATGGGGTTGGCATGG | 50 |
| 51 | TTTGGTGAAAAACGCACGAGGATACCCAGTCACTGTGGCCATCATCCTGG | 100 |
| 101 | ATNGTGTNCTTGCACTCGGCCAGANTGTTCCNCAGTTTGATGGTCTTGTC | 150 |
| 151 | ACGGGAGCCACACACAATTTGACGGTTGTCCACTGAGAATGCCACGGAGA | 200 |
| 201 | GCACATCCTTAGTATGGTCTTCGAAACGCCTGGTGGTCTTTCCGGCGGCG | 250 |
| 251 | ANATCCACAAACGCAGGGTCNTGTCCCACTCCACGCAGANATANTAA | 298 |

Figure 2.32. Nucleotide sequence of PCR product obtained with degenerate primers on first strand cDNA. The letter N, represents any nucleotide.

| | |
|---|---|
| 1) <i>C. reinhardtii</i> Cblp gene for a G protein beta subunit | |
| ACCESSION NUMBER X53574 | |
| 72.1% identity in 136 bp overlap | |
| exp | 289 279 269 TTANTATNTCTGCGTGG--AGTGGGACANGACCC |
| crgplp | AGGACGTGGTCATCTCCTCCGATGGCCAGTTCTGCCTGACTGGCTCGTGGGACGGCACCC |
| 670 680 690 700 710 720 | |
| exp | 259 249 239 229 219 209 TGCGTTTGTGGGATNTCGCCGCCGAAAGACCACCAGGCGTTTCGAAGACCATACTAAGG |
| crgplp | TGCGTCTGTGGGACCTGAACACCGGCACCACCACCGCGCTTCGTGGGCCACACCAAGG |
| 730 740 750 760 770 780 | |
| exp | 199 189 179 169 159 149 ATGTGCTCTCCGTGGCATTCTCAGTGGACAACCGTCAAATTGTGTGTGGCTCCCGTGACA |
| crgplp | ATGTGCTGTCCGTGGCTTTCTCGGTGGACAACCGCCAGGTGGGGCGGCAAGGCCAAGGCG |
| 790 800 810 820 830 840 | |
| exp | 139 129 119 109 99 89 AGACCATCAAACCTGNNGAACANTCTGGCCGAGTGCAAGNACACNATCCAGGATGATGGCC |
| crgplp | AAGGAGGGGGGAGGTCGGCAGGCGGGGTAGCGCGTGGATGCCCGGCGGAGGAAACCACCG |
| 850 860 870 880 890 900 | |


```

2) N. crassa cpc-2 gene
ACCESSION NUMBER X81875

68.9% identity in 286 bp overlap

exp          289      279      269
              TTANTATNTCTGCGTGGAGTGGGACANGACCCTGCG
              |:||||| | - ||||| |:||||| ||
nccpc2 AGGTGATCTCTTCCGACGGTGCCTACGCCCTCTCTGCCT--CGTGGGACAAGACCCTCCG
          2320      2330      2340      2350      2360      2370

exp          259      249      239      229      219      209
              TTTGTGGGATNTCGCCGCCGGAAGACCACCAGGCGTTTCGAAGACCATACTAAGGATGT
              | | ||||| :| | | ||||| | ||||| | ||||| | | | | | | | | | |
nccpc2 TCTCTGGGAGCTTTCCACCGGCACCACCACCCGCCGTTTCGTCGGCCACACCAACGACGT
          2380      2390      2400      2410      2420      2430

exp          199      189      179      169      159      149
              GCTCTCCGTGGCATTCTCAGTGGACAACCGTCAAATTGTGTGTGGCTCCCGTGACAAGAC
              ||||| | | ||||| | ||||| | | | | | | | | | | | | | | | |
nccpc2 TCTCTCCGTCTCTTTCTCCGCCGACAACCGCCAGATCGTCTCCGGTTCGCGGATCGTAC
          2440      2450      2460      2470      2480      2490

exp          139      129      119      109      99      89
              CATCAAACTGNNGAACANTCTGGCCGAGTGCAAGNACACNATCCAGGATGATGGCCACAG
              ||||| |:|||||: || | | |||||: ||: || | | |||||
nccpc2 CATCAAGCTGTGGAACACCCTCGGTGACTGCAAGTTCACCATCACCGAGAAGGGCCACAC
          2500      2510      2520      2530      2540      2550

exp          79      69      59      49      39      29
              TGACTGGGTATCCT--CGTGCGTTTTTACCAAACCATGCCAACCCATTATTGTNTCC-G
              || ||||| |||| | || | | |||| | |||| | ||||| | |||||
nccpc2 CGAGTGGGTTTCTGCGTCCGCTTCAGCCCAACCCCAAGAACCCCGTCATTGTCTCCTC
          2560      2570      2580      2590      2600      2610

exp          19      9
              CTAAGTGGGAAAATATCTGCTGTTC
              | | ||||| | | |
nccpc2 CGGCTGGGACAAGCTCGTCAAGGTATGTTGTATAACCTGCTTTACTCCCCCCCCAAAAA
          2620      2630      2640      2650      2660      2670

```

Figure 2.33. Alignment of PCR product obtained with degenerate primers on cDNA with non-nAChR sequences. Sequences shown represent the two highest nucleotide identities obtained from searching the EMBL database using the gcg program Fasta (Pearson and Lipman, 1988). Exp = experimental.

In summary, PCR on circularised genomic DNA and circularised cDNA has resulted in the isolation of two fragments, encoding the missing N-terminus or part of the missing N-terminus, (a and b respectively), from fragment c. Together, the fragments represent an α -like subunit of the nAChR from *Manduca*. The sequence includes some 5' untranslated region, the complete deduced coding region with putative signal sequence peptide and the complete 3' untranslated region. The sequence of the α -like subunit, named MARA1, is shown in Figure 2.34. Features that are indicative of an α -like subunit of the nAChR are highlighted and are discussed in section 2.5.

| | | |
|------|--|------|
| 1 | GTGCGAGGCCCAACGTGTGGAGTTGGGCGTCAACTCATGCGGAGTGTGACAAATATTACTTACATGGTGTGTT | 75 |
| -21 | M R S V T K Y Y L H G V V | -9 |
| 76 | TTGTTTTCGACAGGATGTGCCGGCAACCCGACGCGAAGCGGCTGTACGACGATCTGCTCAGCAATTACAACAAG | 150 |
| -8 | L F A T G C A G N P D A K R L Y D D L L S N Y N K | 17 |
| 151 | TTGGTGCGGCCTGTACTCAACGTGACGCGACGCGCTCACCGTGCGCATCAAGCTCAAGCTGAGCCAGCTCATCGAC | 225 |
| 18 | L V R P V L N V S D A L T V R I K L K L S Q L I D | 42 |
| 226 | GTGAATCTCAAGAATCAAATAATGACAACCAATCTATGGGTGGAACAGAGCTGGTACGACTACAAGCTTTCGTGG | 300 |
| 43 | V N L K N Q I M T T N L W V E Q S W Y D Y K L S W | 67 |
| 301 | GAGCCGCGCGAGTACGGCGGCGTGGAAATGCTGCACGTGCCTTCCGACCACATCTGGCGGCCGACATCGTGCTT | 375 |
| 68 | E P R E Y G G V E M L H V P S D H I W R P D I V L | 92 |
| 376 | TACAACAATGCTGACGGCAACTTTGAGGTGACGCTGGCGACTAAGGCGACGCTCAATTACACCGGCCGAGTGGAG | 450 |
| 93 | Y N N A D G N F E V T L A T K A T L N Y T G R V E | 117 |
| 451 | TGGCGTCCGCCCCCATCTACAAGTCTCGTGCGAGATCGACGTAGAGTACTTTCCATTGACACGACAGACGTGC | 525 |
| 118 | W R P P A I Y K S S C E I D V E Y F P F D Q Q T C | 142 |
| 526 | GTCATGAAGTTTGGCTCGTGGACCTACGACGGGTTTCAGGTAGATCTGCGACATATCGATGAAGTGCAGGCACC | 600 |
| 143 | V M K F G S W T Y D G F Q V D L R H I D E V R G T | 167 |
| 601 | AACGTCGTGGAGTTGGGCGTCGACCTCTCCGAGTTTACACCTCTGTGAGTGGGACATCCTGGAGGTGCCCGCC | 675 |
| 168 | N V V E L G V D L S E F Y T S V E W D I L E V P A | 192 |
| 676 | GTCCGAAATGAAAAGTTCTACACGTGCTGCGACGAGCCCTATCTAGATATAACTTTCAACATCACGATGCGCCGC | 750 |
| 193 | V R N E K F Y T C C D E P Y L D I T F N I T M R R | 217 |
| 751 | AAAACACTCTTCTACACCGTTAATCTGATCATTCGTCGATGGGAATATCGTTCCTGACGGTCCCTGGTATTTTAC | 825 |
| 218 | K T <u>L F Y T V N L I I P C M G I S F L T V L V F Y</u> | 242 |
| 826 | TTGCCTTCGGACAGTGGCGAGAAGGTATCGCTGTCCATATCGATCCTACTGTGCTCACCGTGTTCCTCTGCTG | 900 |
| 243 | <u>L P S D S G E K V S L S I S I L L S L T V F F L L</u> | 267 |
| 901 | TTGGCCGAGATCATTCACCGACTTCGCTGGTGGTGCCACTGCTTGGCAAGTTTGTCTCTTCACCATGATCCTC | 975 |
| 268 | <u>L A E I I P P T S L V V P L L G K F V L F T M I L</u> | 292 |
| 976 | GATACGTTTCAGCATATGCGTGACTGTGGTGGTATTGAATGTGCACTTCCGCTCGCCACAGACGCACACAATGTG | 1050 |
| 293 | <u>D T F S I C V T V V V L N V H F R S P Q T H T M S</u> | 317 |
| 1051 | CCGTGGGTGCGGCGCGTGTTCATCCACGTGCTGCCGCGGCTCCTGGTCATGCGCGCCCGCACTACCGACTCGAC | 1125 |
| 318 | P W V R R V F I H V L P R L L V M R R P H Y R L D | 342 |
| 1126 | CCGCACCGCAGTCGCTTCGACGAGTGGTGACCGCGCTGGCGAGACCACACTATGGGACGAGGGCTCGCCGGGC | 1200 |
| 343 | P H R S R F A G L V T G A G E T T L W D E G S P G | 367 |
| 1201 | GTGCCGCTCCGCCCCGTCCGCGCCCTGCGACCCCCGCTGGCGCCCTGCGCCGCTGCGCGCCGCTGAGGCG | 1275 |
| 368 | V P A P P R P P P C A P P L A P C A A C A P A E A | 392 |
| 1276 | CCGGCGCTCTGCGACGCGCTGCGCCGGTGGCACCGGTGTCCCAGCTACATAAGGCTATTGACGGCATCAATTAT | 1350 |
| 393 | P A L C D A L R R W H R C P E L H K A I D G I N Y | 417 |
| 1351 | ATTGCCGACCAAACTAGGAAAGAGGAAGAGTCCACTAGGGTGAAAGAGGACTGGAAGTACGTAGCGATGGTACTC | 1425 |
| 418 | I A D Q T R K E E E S T R V K E D W K Y V A M V L | 442 |
| 1426 | GACAGACCATTCCTGTGGATATTACGCTAGCGGTGGTGGTGGGCTCGGCGGGCATCATCCTGACGGCGCCGACG | 1500 |
| 443 | D R <u>P F L W I F T L A V V V G S A G I I L</u> Q A P T | 467 |
| 1501 | CTGTACGACGAGCGCGGCCCATCGACGTGCGCCTGTGCGAGATAGCGTACACGCCGCCAAGCCGCGCCACCG | 1575 |
| 468 | L Y D E R A P I D V R L S E I A Y T A A K P R P P | 492 |
| 1576 | CCGCCACGCTGAGCGGGTCTCGCGTCACGTGCTACGTGTACGCGTGACGCGTCACGCATCACGCGTCACGCGTC | 1650 |
| 493 | P P R | 495 |
| 1651 | GGCGTCTCTCGACGGGCTCCAGTGATTCTCGCTCGTATTTTCGATTACGCAAATTCGAATTTGCAATATTGTAC | 1725 |
| 1726 | ATAGTATACATAATTATTATAGTTTATACAAAGAAACACCGCGATCGATTCTAAGTCGAATTTCTTTTGATAGAG | 1800 |
| 1801 | ACGAAATAAAATGATCGTTTGTAT | 1824 |

Figure 2.34. Nucleotide and amino acid sequence of MARA1. By analogy with other insect α -like subunits (Gundelfinger, 1992), putative transmembrane domains (underlined amino acids), the cys loop (italicised amino acids) and the vicinal cysteines (bold C) are shown. Predicted N-glycosylation sites are shown (bold N).

2.5 Discussion

2.5.1 Method analysis

A number of molecular biology techniques were used to determine the complete coding sequence of an α -like subunit of the nAChR from *Manduca*.

The cDNA library screen did not result in a full-length MARA1 clone and thus did not contribute to the sequence data despite the screening of approximately 1.5 million clones. As a cDNA library is representative of the mRNA present in the original preparation, this suggests that either

1) the cDNA is of inferior quality as a result of either degradation of RNA before or after isolation; poor quality reverse transcriptase and inappropriate reaction conditions in first strand cDNA synthesis or badly controlled second strand cDNA synthesis.

2) The cDNA for MARA1 was in such low abundance that the likelihood of finding the full-length clone was statistically very low.

or 3) The library screen was in some way at fault.

In order to determine the quality of the manufacturer's cDNA, the library could be screened for an ubiquitous DNA such as actin. If this was found in a truncated form, it would indicate that in general the cDNAs in the library would not contain the entire nucleotide sequence of the corresponding mRNA. As the information about the preparation of the customised library is limited, any of the reasons given in 1) above could be at fault. Indeed, the presence of truncated cDNA probably encoding an α -like subunit of the nAChR in the library was confirmed with nested PCR (see Figure 2.4).

The quality of the library screen could be assessed using a more rigorous MARA1 control, which was not contemplated for reasons of time and cost. This would entail subcloning fragment c into the same vector as the library, i.e. λ ZapII (which itself would involve combining the insert with left and right arms of the λ ZapII to get a recombinant phage and then *in vitro* packaging of the recombinant phage into mature phage particles) and then plating out as before so that every screening step could be compared to that used for the library. However, the excessive cost and time which would be spent on this experiment did not justify its role as a control.

Inverse PCR on double stranded, circularised cDNA was successful in that more DNA sequence data was obtained from the missing N-terminal end of fragment c. The predicted amino acid sequence now appeared to be lacking only the extreme N-terminal

amino acids (approximately 57-77 residues by comparison with other insect α -like subunits, see alignment, Figure 2.36). Ligation anchored PCR failed to add any further sequence data while inverse PCR on genomic DNA resulted in a DNA sequence that encodes the missing N-terminus from fragment c.

Common to both cDNA requiring PCR strategies is the synthesis of first strand cDNA. cDNA clones are sometimes truncated, containing only 3' untranslated and varying amounts of the protein coding sequence. This is due to the lack of generation of full-length cDNA strands during the reverse transcription reaction. As poly [A+] RNA instead of total RNA was used in both cases, contamination by non-specific products is improbable. Plausibly, the reverse transcriptase dissociated from the poly [A+] RNA at GC rich regions denoting areas of complex secondary structure, resulting in truncated cDNAs, which, by their very nature were not amplified in the PCR.

It is apparent that as the same antisense primer (antisense 3) was used in both cases and resulted in fragment b for the inverse PCR strategy, the lack of results in the ligation anchored PCR strategy must be due to either a different factor or a combination of factors.

Comparison of the sequence of the anchor oligonucleotide with other sequences in the Genbank and EMBL databases using the gcg program Fasta (Pearson and Lipman, 1988) indicated little potential for the anchor oligonucleotide to anneal non-specifically to the cDNA, as the sequences extracted from the database were mainly from plants. Also, radiolabelling of the HPLC purified anchor oligonucleotide, followed by gel electrophoresis and autoradiography did not reveal any degradation of the oligonucleotide. Therefore, it was doubtful that synthesis of the anchor oligonucleotide was the problem.

The ligation reaction is dependent on the first strand cDNA having hydroxyl groups at both termini, leaving only the 3' end available for ligation, and on the 3' block of the anchor oligonucleotide, leaving only the 5' end available for ligation. As these pre-requisites were adhered to, and as the reaction conditions were set according to studies developed by Tessier *et al.* (1986), this leaves the effect of acceptor:donor ratio, i.e. the ratio of anchor oligonucleotide to single stranded cDNA. For ligation of oligodeoxyribonucleotides, Tessier *et al.* reported that a ratio of one molecule of donor to one molecule of acceptor only resulted in 3% ligation efficiency, but increasing the amount of acceptor to 5 molecules resulted in 17% ligation efficiency. As the amount of

cDNA in the ligation reaction was estimated, it is possible that the amount of cDNA present never reached the threshold level that was critical for ligation.

Searching for a full-length MARA1 clone

The 90bp overlap between fragments a and c implies that they are from the same α -like subunit. This was confirmed using restriction digest analysis on *Manduca* genomic DNA, which gave a similar pattern of bands for each fragment, and by PCR that spliced part of each of the two fragments together by overlap extension.

Three approaches were applied to the construction of a full-length clone of MARA1. The first involved the attempted formation of a *de novo* copy of a full-length clone using the sequence information gained from fragments a and c to design specific primers to the 5' and 3' ends of the subunit, while the second involved the utilisation of an engineered restriction site to ligate fragments a and c together. The final approach searched for a genomic clone.

There were several advantages to making a *de novo* copy of the full-length cDNA clone. Firstly, ligation of the two fragments would lead to an hybrid molecule composed of genomic DNA and cDNA, with only the first part of the extracellular region containing introns - in contrast a *de novo* copy would comprise entirely cDNA. Secondly, as no convenient restriction sites were available for the ligation of the two fragments in their overlapping regions, the introduction of a novel restriction site by PCR would be necessary, making the ligation approach more difficult. Moreover, this could lead to the introduction of more mutations (in addition to the engineered restriction site) in the sequence due to the increase in error rate inherent with an increase in cycles of PCR. This would necessitate the sequencing of numerous clones to identify one without mutations (reviewed in Frohman, 1993). Contrastingly, PCR under inefficient but low error rate conditions using a minimum number of cycles and specific primers designed to the extreme N- and C-terminals of MARA1 could amplify a new cDNA likely to be free of mutations (Eckert and Kunkel, 1990). Thirdly, although unlikely, it could be possible to join two fragments that are never found together *in vivo*, if alternate promoters, splicing, polyadenylation signal sequences or highly homologous subunits resulted in multiple 5' and 3' ends - using specific primers to the extreme N- and C-terminals of MARA1 would confirm that the resulting amplified cDNA represents an mRNA that is present in the starting population (Ragsdale *et al.*, 1989). Finally, a *de novo* copy of a cDNA clone would facilitate injection of the subunit into *Xenopus* oocytes as the DNA could be injected directly or transcribed into cRNA, an option not available when using genomic

DNA which has to be injected into the nucleus of the oocyte. A full-length MARA1 clone was neither found *de novo* nor constructed from its constituent fragments.

2.5.2 Possible causes for the lack of a full-length MARA1

The extreme difficulty in isolating a full-length cDNA clone of MARA1 indicates either a very rare message or a large degree of RNA secondary structure. Interestingly, similar problems have been found with the isolation of a putative β -like subunit encoding an nAChR from *Manduca*, MARB1 (*Manduca* Acetylcholine Receptor Beta-like subunit 1; J.Eastlake, University of Bath) which is also incomplete at the N-terminus.

Considering that ACh is the major neurotransmitter in the insect CNS (Colhoun, 1963; reviewed in Sattelle, 1980), and that nicotinic AChRs are known to be more abundant than muscarinic AChRs in the insect CNS (reviewed in Breer and Sattelle, 1987), a particularly rare message encoding an α -like subunit of the nAChR in *Manduca* seems unlikely. Nevertheless, an excessive number of PCR cycles (over 40) were necessary to isolate an internal fragment of MARA1. Amplification of the appropriate product normally requires cDNA in very low quantities, suggesting that the mRNA for MARA1 is very rare. Interestingly, the search for a full-length muscarinic AChR using cDNA from *Manduca* has also been fruitless: only two partial length fragments have been isolated from genomic DNA - the remainder of the sequence and the full-length clone have not been forthcoming (A.Wang, personal communication), indicating that it too is in extremely low abundance.

The question of secondary structure has been addressed using a variety of methods. In attempts to destroy the putative secondary structure by heat, the incubation temperature of the reverse transcription reaction was increased from a single hour at 37°C to a ramped protocol using temperatures of 37°C, 42°C and 50°C for 30min each. In addition, a thermostable Reverse Transcriptase that operated at temperatures of 70°C was used. Neither of these procedures solved the problem. The addition of methylmercury hydroxide (Payvar and Schimke, 1979) which is known to disrupt secondary structure was considered, but discounted as being too dangerous.

The gcg program mfold based on the method of Zuker (1989) was used to predict optimal and suboptimal RNA folding. Only the extracellular region of MARA1 was considered as a) this is the region in which all cDNA transcripts appear to be truncated and b) the program uses a 'combinatorial' method which forms structures by combining

all potential helices in all possible ways, such that the computation time increases with the length of the sequence.

For MARA1, optimal and suboptimal secondary structures were predicted, as uncertainties in the folding model and the folding energies do not necessarily result in correct folding for the optimal structure.

The optimal structure obtained, Figure 2.35 uses an energy minimum of -159.1 kcal/mol. A suboptimal structure with an energy of -158.6 kcal/mol differs only in its representation of the extreme N-terminal nucleotides, equivalent to the amino acids asparagine 1 to threonine 106. In both structures, base pairing is extensive (as seen by the long stems) and hairpin loops are small, suggesting a highly folded region of the molecule.

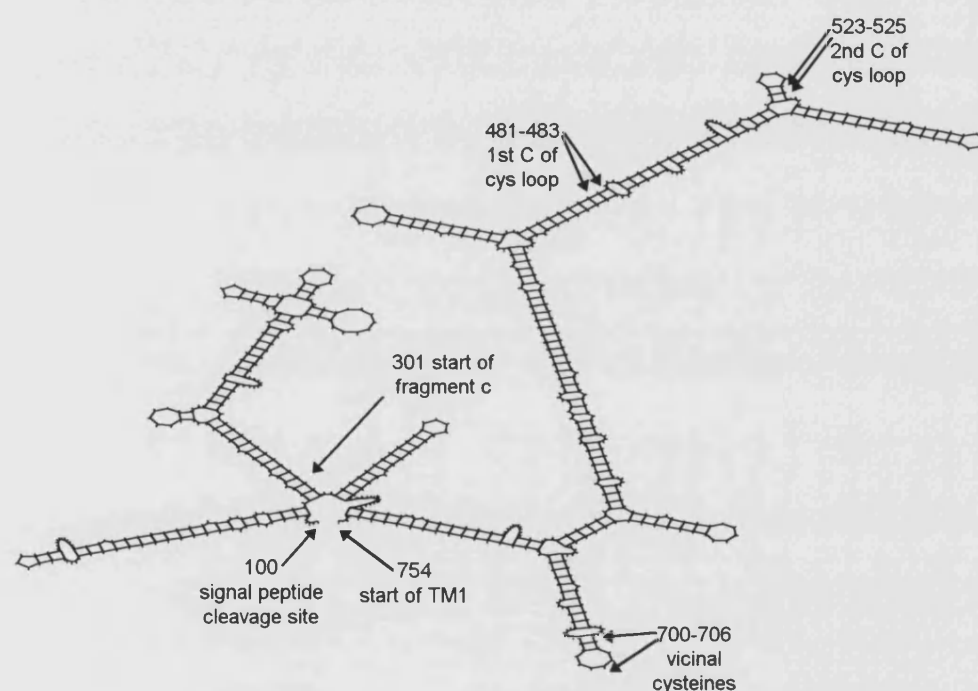


Figure 2.35. Optimal RNA secondary structure prediction for the extracellular region of MARA1. Structure predicted using the gcg program mfold (Zuker, 1989).

The 5' end of fragment c is at nucleotide position 301 (indicated on Figure 2.35). The mRNA sequence upstream of nucleotide 301 represents the extreme N-terminus and shows very tight folding, due to closely spaced hairpin loops. Moreover, the mRNA in the extracellular region comprising nucleotides 100-301 is GC rich: guanine and cytosine nucleotides represent 53% of the total nucleotides within this region. Thus, this analysis helps explain the difficulties incurred in isolating the N-terminus of MARA1.

In contrast, the same analysis of the extracellular region of *Drosophila* ALS shows relatively larger hairpin loops in both optimal and suboptimal structures. For the optimal structures, a maximum of 8 nucleotides in an hairpin loop is seen in MARA1, compared with a maximum of 25 in ALS, suggesting that the extracellular region of ALS is less tightly folded.

2.5.3 Analysis of the MARA1 sequence

To facilitate comparison, an amino acid alignment between *Manduca* MARA1 and the insect α -like subunits from *Drosophila* (ALS and D α 2/SAD, the latter referred to as SAD on alignment) and *Schistocerca* (α L1 but referred to as ARL2 on alignment) is shown in Figure 2.36.

For MARA1, the 5' untranslated sequence of 36 nucleotides is part of a 5' leader sequence of unknown size. In *Drosophila*, these 5' leader sequences vary in length from 1282 nucleotides in ALS (Bossy *et al.*, 1988) to only 343 nucleotides in D α 2/SAD (Sawruk *et al.*, 1990a). Comparison with *Schistocerca* α L1 is not possible as only a partial length leader sequence of 20 nucleotides has been reported (Marshall *et al.*, 1990). These observations suggest that MARA1 may be missing a substantial number of nucleotides from its 5' leader sequence. Moreover, the genomic sequence of fragment a (Figure 2.16) does not contain any nucleotides resembling a TATA box, that would help locate the transcription start site.

The open reading frame of 1548 nucleotides encodes 516 amino acids, the first 21 of which have the features of a putative signal peptide, being particularly hydrophobic (11 out of 21 residues). The first residue of the mature protein is probably asparagine 1 (nucleotide position 100) as this conforms to von Heijne's rules (1983, 1986) and is consistent with predicted signal cleavage sites for other insect α -like subunits of the nAChR.

By analogy with other insect α -like subunits (Figure 2.36), the first 219 residues of the N-terminus represent the predicted extracellular region of the α -like subunit. The sequence CEIDVEYFPFDQQTC at positions 128 to 142 corresponds to the cys loop - a structural motif present in all subunits of the nAChR and the two cysteines at positions 201 and 202 correspond to the vicinal cysteines definitive of α -subunits. As these vicinal cysteines are found at positions 192 and 193 in *Torpedo* (the prototype of the nAChR; Noda *et al.*, 1982; see Appendix 1), but at positions 201 and 202 in insects studied to

date, due to a nine residue insertion from positions 164 to 172 inclusive, MARA1 is designated an α -like subunit of the nAChR. The cys loop has been indicated in the assembly of the subunits of the nAChR: it has been demonstrated that cysteine 128 and 142 form a disulphide bridge in each subunit to maintain the correct conformation of the extracellular region of the nAChR (Mishina *et al.*, 1985). Although computer modelling studies suggest that the cys loop may be a major determinant of agonist binding (Cockcroft *et al.*, 1990), there appears to be little experimental evidence to support this hypothesis. In contrast, the vicinal cysteines are well documented as part of the agonist binding site (reviewed in Devillers-Thiéry *et al.*, 1993) and will be discussed further (see Agonist and antagonist binding).

Analysis of the mature protein using the gcg program Motifs gave potential N-glycosylation sites (Pless and Lennarz, 1977). Glycosylation of an asparagine residue appears to be the only post translational modification that affects ligand binding, as shown for the α -btx binding site in *Torpedo* (Mishina *et al.*, 1985). As protein folding plays an important role in the regulation of N-glycosylation, it is likely that only the extracellular region contains N-glycosylation sites. Potential N-glycosylation sites (corresponding to the consensus pattern of (N) X (T,S) where "X" represents any amino acid) in the extracellular region of MARA1 are found at amino acid positions 24, 111 and 212. The first and last are also predicted for ALS (Bossy *et al.*, 1988), D α 2/SAD (Sawruk *et al.*, 1990a) and α L1 (Marshall *et al.*, 1990). The site at position 111, although not present in the other insect α -like subunits, is present at equivalent positions in chick α 7 and α 8 subunits (Couturier *et al.*, 1990b; Schoepfer *et al.*, 1990).

Following the extracellular region are three putative transmembrane domains (TM1-3), a putative intracellular loop and a fourth putative transmembrane domain (TM4) before the C-terminus (by analogy with other insect α -like subunits; see Figure 2.36 and Gundelfinger, 1992).

The transmembrane domains are predominantly hydrophobic and exhibit high homology with other insect α -like subunits (for example, 96%, 92% and 88% for TM1 with ALS, D α 2/SAD and α L1 respectively). By analogy with other nAChR subunits, it is predicted that TM2 lines the ion channel. Comparison with *Torpedo* subunits (see Figure 1.3; Noda *et al.*, 1983b; Devillers-Thiéry *et al.*, 1993) indicates the presence of essential residues that putatively form the superimposed rings within the ion channel (see section 1.2.7). Negatively charged, aspartate and glutamate residues are present in the inner, intermediate and outer rings, consistent with subunits of the nAChR from *Torpedo* (Noda

et al., 1983b) and may be involved in ion selectivity (reviewed in Bertrand *et al.*, 1993). Serine and leucine residues corresponding to the serine and leucine rings are also present, however a serine residue is present in the threonine ring, contrasting with the α and γ subunits of *Torpedo* but consistent with the β and δ subunits of *Torpedo* (Noda *et al.*, 1983b). Serine and leucine rings control ion channel gating whereas the threonine ring helps control ion size selectivity. Interestingly, analysis of the sequence encoding the putative TM2 from MARB1 shows that it contains a threonine residue at the site of the threonine ring and it is therefore possible that the β -like subunit in *Manduca* controls ion size selectivity.

The intracellular loop, thought to be the cytoplasmic region of the subunit, shows pronounced variability. Most striking, is the shortness of the intracellular loop in MARA1 compared with other α -like subunits from insects - it is 140 amino acids in length and therefore 47 residues shorter than ALS, 40 residues shorter than D α 2/SAD and 41 residues shorter than α L1.

Analysis of the mature protein using the gcg program Motifs gave potential phosphorylation sites within the intracellular loop. Phosphorylation does not appear to affect ligand binding although it has been demonstrated to be involved in receptor desensitisation and subunit assembly (reviewed in Huganir and Greengard, 1990; Green *et al.*, 1991). There are several potential phosphorylation sites in the cytoplasmic loop. Potential tyrosine phosphorylation sites are found at amino acid positions 417 and 437, neither of which are predicted for the other insect α -like subunits. These fit the consensus pattern of (R,K) X {2,3} (D,E) X {2,3} Y, where "X" represents any amino acid (Patschinsky *et al.*, 1982). Potential protein kinase c phosphorylation sites are found at amino acid positions 422 and 428 but not in the other insect α -like subunits. These fit the consensus pattern of (S,T) X (R,K), where "X" represents any amino acid (Kishimoto *et al.*, 1985; Woodget *et al.*, 1986). However, it is known that the presence of additional basic residues at the N or C terminal of the target amino acid increases the maximal velocity of the phosphorylation reaction (Woodget *et al.*, 1986) and instead these target positions are both close to several acidic glutamate residues, suggesting that they may not be true phosphorylation sites. Interestingly, Green *et al.* (1991) demonstrated that phosphorylation of unassembled γ subunits led to an increase in assembly of all four subunits in cell lines expressing *Torpedo* nAChRs. As the γ subunit is generally considered to play a more structural role than the α subunit which is predominantly ligand binding, phosphorylation of the α -like subunit in MARA1 may be concerned with desensitisation rather than subunit assembly.

The coding region is stopped by the codon TGA and a 3' untranslated region of 240 nucleotides follows which contains the putative polyadenylation consensus signal AATAAA, 14 nucleotides upstream of the polyadenylate tail.

| | | | | | | | | |
|--------------|-------|--|---|---|--|--------------------------------|------------------------------------|--------------------|
| | | | 1 | 50 | | | | |
| Drosophila | SAD | MAPG | CTTRPRPIALLAHIWRHCKPLCLLLVLLLLCETVQANPDAKRLYDDLLSNYNRLIRPVSNN | TDTVLVKLGRLLSQLIDLNLKDQILTTNVWLEHE | | | | |
| Schistocerca | ARL2 | | MAAALP...PMLLLLLLLLLLHHPAAANPDAKRLYDDLLSNYNRLIRPVSNN | TDTVLVKLGRLLSQLIDLNLKDQILTTNVWLEHE | | | | |
| Drosophila | ALS | | MGSVLFAAVFIALHFATGGLANPDAKRLYDDLLSNYNRLIRPVGNN | SDRLTVKMGLRLSQLIDVNLKNQIMTTNVWVEQE | | | | |
| Manduca | MARA1 | | MRSVTKYYLHGVLVFATGCAGNPDAKRLYDDLLSNYNKLVRPVLNV | SDALTVRIKLKLSQLIDVNLKNQIMTTNLWVEQS | | | | |
| | | | 100 | 150 | | | | |
| Drosophila | SAD | WQDHKFKWDP | SEYGGVT | ELYVPSEHIWL | PDIVLYNNADGEYVVT | TMTKAILHYTGKVVWTPPAIFKSS | CEIDVRYFPFDQQT | CFMKFGSWTYDGDQIDLK |
| Schistocerca | ARL2 | WQDHKFRWDPAEYGGVT | ELYVPSEHIWL | PDIVLYNNADGEYVVT | TMTKAVLHHTGKVVWTPPAIFKSS | CEIDVRYFPFDQQT | CFMKFGSWTYDGDQIDLK | |
| Drosophila | ALS | WNDYKLKWN | PDDYGGVDTLHVP | SEHIWHP | PDIVLYNNADGNYEVT | IMTKAILHHTGKVVWKPPAIYKSF | CEIDVEYFPFDEQT | CFMKFGSWTYDGYMVDLR |
| Manduca | MARA1 | WYDYKLSWE | PREYGGVEMLHVP | SDHIWRP | PDIVLYNNADGNFEVTLATKATLNYTGRVEWR | PPAIYKSS | CEIDVEYFPFDQQT | CFMKFGSWTYDGFQVDLR |
| | | | 200 | 250 | | | | |
| Drosophila | SAD | HISQKNDKDNKVEIGIDLREYYPSVEWDILGVPAERHEKYYP | CCAEPYPDIFFNITLRRKTLFYTVNLIIP | CVGISYLSVLV | FYLPADSGEKIALCISIL | | | |
| Schistocerca | ARL2 | HINQKYD.DNKVKVGIDLREYYPSVEWDILGVPAERHEKYYP | CCAEPYPDIFFNITLRRKTLFYTVNLIVP | CVGISYLSVLV | FYLPADSGEKIALCISIL | | | |
| Drosophila | ALS | HLKQTADSDN.IEVGIDLQDYYISVEWDIMRVP | PAVRNEKFYSCCEEPYLDIVFNLTLRRKTLFYTVNLIIP | CVGISFSLSVLV | FYLPSDSGEKISLCSISIL | | | |
| Manduca | MARA1 | HIDEVRGTNV.VELGVDLSEFYTSVEWDILEVPAVRNEKFYT | CCDEPYLDITFNITMRRKTLFYTVNLIIP | CMGISFLT | TVLVFYLPSDSGEKVSLCSISIL | | | |
| | | | 300 | 350 | | | | |
| Drosophila | SAD | LSQTMFFLLISEIIPSTSLALPLL | GKYLLFTMLLVGLSVVITIIILNIHYRKPSTHKMRPWIRSFFIKRLPKLLLMRV | PKDLLRDLA.ANKI...NYGLK | | | | |
| Schistocerca | ARL2 | LSQTMFFLLISEIIPSTSLALPLL | GKYLLFTMVLVGLSVVITIMVLNVHYRKPSTHKMAPWVRKVFI | RRLPKLLLMRVPEQLLADLA.SKRLLRHAHNSK | | | | |
| Drosophila | ALS | LSLTVFFLLLAIEIIPPTSLTVPLL | GKYLLFTMMLVTL | SVVVTIAVLNVNFRSPVTHRM | APWVQRLFQILPKLLC | IERPKKEEPEEDQPPEVLT | DVYHLP | |
| Manduca | MARA1 | LSLTVFFLLLAIEIIPPTSLV | VPLL | GKFVLF | TMILDTFSICVT | VVVLNVHFRSPQ | HTMSPWVRRVFIHVLPRLLVMRRPH.....YRLD | |
| | | | 400 | 450 | | | | |
| Drosophila | SAD | FSKTKFGQALMDEMOMNSGGSSPDSLRR..... | MQGRVGAGGCNGMHVTTATNRFSGLVGALGGGLSTLSGYNGLPSVLSGL | DDSLSDVAAR | | | | |
| Schistocerca | ARL2 | LSAA...AAA | AVAAAASSSAASSPDSL | RHHHLHQHQHHLQLHHLQ | RPGGCNGLH..SATNRF | GGSAGAFG.....GLPSVV | GLDGSLSDVATR | |
| Drosophila | ALS | PDVDKFVNY...DSK | RFSGDYGIPALPASHRFDLAAAGGISAH | CAEPPLPSSLPLPGADDDLFSP | SGLNGDISPGCC.....PAAAAAAAADL | SPTFEK | | |
| Manduca | MARA1 | PHRSR | FAGL.....VTGAGETTLWDEG | SPGVPAPPRPPPCAPPL.....APC.....AACAPAEAPAL | CDALRR | | | |
| | | | 500 | 550 | | | | |
| Drosophila | SAD | KKYPFELEKAIHNV | FIQHMQRQDEFNAEDQDWGFVAMVMDRLFLWLF | MIASLVGT | FVILGEAPSLYDDTKAIDVQLSDVAKQIYNL | TEKKN | | |
| Schistocerca | ARL2 | KKYPFELEKAIHNV | LFIQNHMQRQDEFDAEDQDWGFVAMVLDRLFLWIFT | IASIVGT | FAILCEAPALYDDTKPIDMELSSVAQQQF | LPDIDF | | |
| Drosophila | ALS | .PYAREMEKT | IEGSRFIAQHVKNKDKFESVEEDWKYVAMVLD | RMFLWIFAIA | CVVGTALIIQAPSLHDQSQPIDILYSKIAKKKFELLKM | SENTL | | |
| Manduca | MARA1 | WHR | PELHKAIDGINYIADQ | TRKEEESTRVKEDWKYVAMVLD | RPFLWIFTLAVVVG | SAGIILQAPTLYDERAPIDVRLSEIA...Y | TAAKPRPPPPR | |

Figure 2.36. Alignment of insect α -like subunits. Constructed using the gcg program Pileup (Feng and Doolittle, 1987; Higgins and Sharp, 1989) with sequences extracted from the Genbank and EMBL databases. SwissProt accession numbers: SAD: p17644; ARL2: p23414; ALS: p09478.

Homology with other α -like subunits

DNA sequence identity between MARA1 and other insect α -like subunit genes was analysed for the coding region using the gcg program Distances (Swofford and Olsen, 1990), using an observed number of substitutions per 100 nucleotides. The following % identities were obtained:

| | D α 2/SAD | α L1 | ALS | MARA1 |
|------------------|------------------|-------------|-----|-------|
| D α 2/SAD | 100 | 72 | 62 | 59 |
| α L1 | | 100 | 63 | 62 |
| ALS | | | 100 | 64 |
| MARA1 | | | | 100 |

Genbank accession numbers used were: D α 2/SAD: x52274; α L1: x55439; ALS: x07194.

Complete amino acid sequence identity between MARA1 and other insect α -like subunits was calculated with the gcg program Distances (Swofford and Olsen, 1990), using an observed number of substitutions per 100 amino acids. The following % identities were obtained:

| | D α 2/SAD | α L1 | ALS | MARA1 |
|------------------|------------------|-------------|-----|-------|
| D α 2/SAD | 100 | 82 | 56 | 54 |
| α L1 | | 100 | 57 | 56 |
| ALS | | | 100 | 62 |
| MARA1 | | | | 100 |

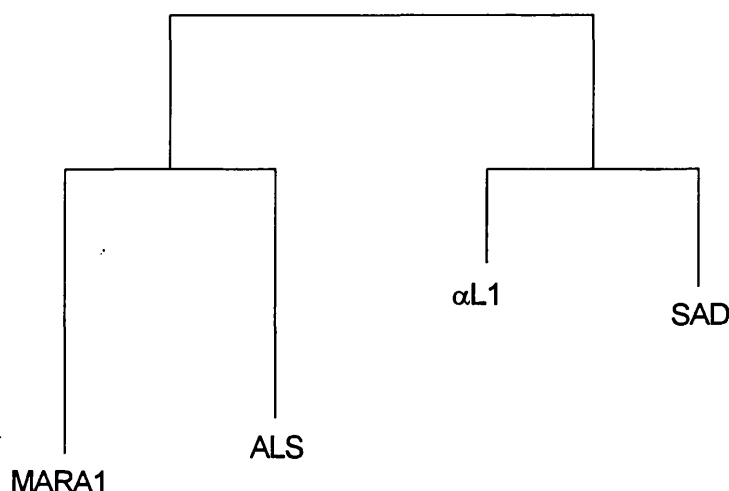
SwissProt accession numbers were: D α 2/SAD: p17644; α L1: p23414; ALS: p09478.

Both nucleotide and amino acid identities show the same pattern between the α -like subunits. Thus, MARA1 shares the highest identity with ALS from *Drosophila*, followed by α L1 from *Schistocerca*, and then by D α 2/SAD from *Drosophila*. D α 2/SAD and α L1 share much greater identity than D α 2/SAD and ALS, implying that they are the homologous α -like subunits of different species.

This is further emphasised by a basic phylogenetic tree, Figure 2.37, constructed using the gcg program GrowTree (Swofford and Olsen, 1990). In order to construct a valid tree, the observed distances used to calculate % amino acid identity quoted above were corrected for multiple substitutions using the Kimura protein method (Kimura, 1983).

This is necessary as the probability that more than one substitution occurred at a single site increases as the time since the sequences diverged increases, resulting in an underestimation of the true distance between all but very closely related sequences.

Figure 2.37. Phylogenetic tree of insect α -like subunits. Constructed using the gcg program GrowTree (Swofford and Olsen, 1990).



The tree shows that D α 2/SAD and α L1 are the least divergent of the four subunits (shown by short vertical lines). MARA1 is the least divergent sequence from ALS but the most divergent sequence of the four subunits (i.e. it has the longest vertical line). It is known that *Drosophila* (Diptera) and *Schistocerca* (Orthoptera) are only distantly related in terms of evolution, whereas *Manduca* (Lepidoptera) and *Drosophila* are very closely related, being members of the division Panorpida (Kristensen, 1981). Therefore, it is rather surprising that D α 2/SAD and α L1 show such high homology but that MARA1 and the *Drosophila* α -like subunits do not. However, a recent evolutionary study on the nAChR (le Novère and Changeux, 1995) suggests that the duplication between ALS and the pair, D α 2/SAD and α L1 is older than the divergence of Orthoptera and Diptera (~300MYA), if it is assumed that D α 2/SAD and α L1 are orthologs, i.e. they have arisen from the 'same' gene in different species. This helps to explain the remarkable similarity between D α 2/SAD and α L1. Furthermore, it can be predicted that the ortholog of ALS also exists in *Schistocerca*.

Although, the Lepidoptera were not included in the study, but assuming that the same case applies whereby duplication of the subunits of the nAChR occurred before divergence of the Lepidoptera and Diptera, it could be predicted that the orthologs of ALS and D α 2/SAD exist in *Manduca*. Given that the amino acid identity between D α 2/SAD and α L1 is 82% and that *Drosophila* and *Manduca* are more closely related than *Drosophila* and *Schistocerca*, it would be expected that an ortholog of ALS or D α 2/SAD would have similarly high identity. However, MARA1 has only 62% amino acid identity with ALS and only slightly less with D α 2/SAD (54%), suggesting that although closely related, it is not the ortholog of either α -like subunit. Furthermore, no other α -like subunits in *Manduca* have been found despite intensive searching. This suggests that only one α -like subunit of the nAChR is present in *Manduca* and therefore that orthologs of the *Drosophila* and *Schistocerca* α -like subunits do not exist in *Manduca*.

Homomeric versus heteromeric receptors

It has been demonstrated that insect α -like subunits can form both homo- and heteromeric nAChR *in vitro*. Breer *et al.* (1985) isolated an homomeric nAChR from *Locusta migratoria*. Reconstitution of this protein in a planar lipid bilayer (Hanke and Breer, 1986) demonstrated that cholinergic agonists activated functional ion channels.

Sawruk *et al.* (1990a) demonstrated that D α 2/SAD cRNA elicited agonist evoked currents when expressed in *Xenopus* oocytes and subjected to voltage clamp recording. High concentrations of ACh produced small inward currents of 10-50nA, while nicotine was able to produce larger currents (~150nA at 20mM) in a concentration dependent manner, giving an EC₅₀ value of 10mM.

α L1 has also been shown to form homomeric nAChRs in the *Xenopus* oocyte expression system (Marshall *et al.*, 1990; Amar *et al.*, 1995). Nicotine produced small currents (20-120nA) in a concentration dependent manner, giving an EC₅₀ value of 0.83mM (Amar *et al.*, 1995). Importantly, these nicotine-evoked currents were blocked by the cholinergic antagonists, α -btx and MLA, demonstrating that α L1, as an homomer produced a functional nAChR (Amar *et al.*, 1995).

However, for both D α 2/SAD and α L1, it is suggested that the atypical size of currents and the high EC₅₀ values for nicotine (considering that both insects are nicotine sensitive) reflect the absence of additional subunits necessary for *in vivo* function in the insect. Co-injection of D α 2/SAD with the *Drosophila* β -like subunit, ARD produced no significant changes in the response profile though, indicating that it was either unlikely to

be the other subunit expressed with D α 2/SAD or that yet another subunit was necessary for more typical expression (Sawruk *et al.*, 1990a).

In both these cases, it may be that small currents are only produced due to inefficient assembly of invertebrate proteins by a vertebrate expression system. This quandary could be solved using an invertebrate expression system such as baculovirus (for an example of the expression of *Drosophila* GABA receptors see Lee *et al.*, 1993) or the *Drosophila* S2 cell line (Millar *et al.*, 1994).

Bertrand *et al.*, 1994, demonstrated that neither ALS nor D α 2/SAD formed functional receptors as homomers (the latter contrasting to data from Sawruk *et al.*, 1990a) but would function when co-expressed with the chick β 2 subunit (but not with ARD). The two *Drosophila* subunits gave different characteristics, EC₅₀ values for ACh being 0.18 μ M and 35 μ M and for nicotine being 1.3 μ M and 7.4 μ M for ALS and D α 2/SAD respectively. ALS/ β 2 was blocked by α -btx whereas D α 2/SAD/ β 2 was not blocked by α -btx. Thus the *Drosophila* subunits ALS and D α 2/SAD do respond to physiological concentrations of cholinergic agonists when co-expressed with an appropriate β -subunit. This suggests that although D α 2/SAD can undoubtedly function as an homomer, it functions better as an heteromer, which may be its native state.

Given that MARA1 has the greatest sequence homology with ALS and that it appears to be more closely related evolutionarily to ALS rather than D α 2/SAD or α L1, it may be expected that it forms part of an heteromeric nAChR. For this to occur, β -like subunits would have to exist in the *Manduca* nervous system. Indeed, a partial length cDNA encoding a putative β -like subunit has been isolated from *Manduca* (MARB1) in this laboratory (J. Eastlake and A. Wolstenholme, unpublished data) indicating that heteromeric receptors for *Manduca* must exist. To determine whether MARA1 and MARB1 can function as subunits in the same nAChR though, will require the generation of full-length cDNAs.

The existence of more than one *Drosophila* α -like and β -like subunit of the nAChR form a precedent for other insects. Currently, three α -like subunits and two β -like subunits have been found in *Drosophila* and one of each has been isolated from *Schistocerca*. Moreover, Hermsen *et al.* (1991) have reported the isolation of four α -like subunits and two β -like subunits from another member of the Orthoptera, *Locusta migratoria* which they suggest form heteromeric nAChRs. However, the experiments reported in this study provide no evidence for the existence of additional subunits in *Manduca*.

Agonist and antagonist binding

The agonist binding site is a pre-requisite for a functional nAChR. Affinity labelling and site directed mutagenesis experiments have resulted in the identification of residues required for ligand binding. The photoactivatable cholinergic ligand, p-N,N-dimethylaminobenzene diazonium fluoroborate (DDF) labels amino acids from three different regions of the N-terminal extracellular region in *Torpedo* (Dennis *et al.*, 1986, 1988; Galzi *et al.*, 1990), suggesting a multiple loop model for cholinergic binding. These are residues tryptophan 86 and tyrosine 93, tryptophan 149 and tyrosine 151, and tyrosine 190, cysteine 192, cysteine 193 and tyrosine 198 (see Appendix 1 and Figure 1.3) which in MARA1 correspond to residues tryptophan 86, tyrosine 93, tryptophan 149, tyrosine 151, tyrosine 199, cysteine 201, cysteine 202 and tyrosine 206 (see Figure 2.34). It is thought that the tyrosines, which show electronegativity attract the quaternary ammonium of ACh.

Site directed mutagenesis of the α subunit of the nAChR and subsequent expression in *Xenopus* oocytes has shown that mutation of cysteine 192 and cysteine 193 in *Torpedo* results in the total loss of ACh evoked responses compared with the wild type receptor. In addition there is a marked decrease in the apparent binding affinity for ACh (Mishina *et al.*, 1985). This indicates that cysteines 192 and 193 are imperative for ACh binding and activation of the receptor. Further experiments using the chick $\alpha 7$ receptor (Galzi *et al.*, 1991) have demonstrated that tyrosine 92, tryptophan 148 and tyrosine 187 (in *Manduca*, corresponding residues are tyrosine 93, tryptophan 149 and tyrosine 199), when mutated to phenylalanine, give a 10-fold (Y92F, Y187F) or 100-fold (W148F) reduction in apparent ACh affinity whereas they give a 2-fold (Y92F, Y187F) or 300-fold (W148F) reduction in apparent nicotine affinity. Therefore, although all these residues are necessary for agonist binding, the two agonists ACh and nicotine do not establish the same interactions with these three residues. It appears that tryptophan 148 is essential for nicotine binding while tyrosine 92 and tyrosine 187 are less crucial. Given these findings, if MARA1 were truly resistant to nicotine and thus gave a different agonist profile to that of nicotine sensitive receptors, it might be expected that at least one of these residues would be replaced with a residue unable to form the correct bonds with nicotine but not necessary or still able to form the correct bonds with ACh. In this respect, tryptophan 148 seems an ideal candidate. More recently, replacement of tryptophan 54 of the chick $\alpha 7$ receptor (corresponding to tryptophan 55 in *Manduca*) by a phenylalanine, alanine or histidine residue has been shown to cause a progressive decrease in binding affinity and in responses for ACh and nicotine (Corringer *et al.*, 1995). Taking nicotine for example, the EC_{50} of the wild type receptor was 4.3 μ M,

whereas mutations gave EC_{50} values of 7.7 μ M for W54F, 45 μ M for W54A and 200 μ M for W54H. The authors suggested that tryptophan 54 was part of a 'complementary component' of the $\alpha 7$ binding site, as opposed to the three loop 'principal component' and was equivalent to the non- α contribution from δ and γ subunits in the muscle receptor (see Chapter 1, section 1.2.7). Analysis of the extracellular region of MARA1, however, reveals that all these residues are present, as shown in Figure 2.38.

Clarke (1991) speculated that glutamate 133 within the cys loop of MARA1 may contribute toward *Manduca*'s nicotine insensitivity. This is based on the ligand binding model of Cockcroft *et al.* (1990) which proposes that the cys loop of the ligand gated ion channel superfamily determines their ligand specificity. However, it should be emphasised that there is no experimental data to support this model. As all nAChR subunits contain this cys loop whereas the α subunit is considered the major ligand binding subunit, it seems unlikely that it plays a predominant role in ligand binding. Interestingly however, the cys loop has been implicated in the binding of allosteric effectors of the nAChR such as physostigmine, which activates the nAChR at low concentrations and blocks the open ion channel at high concentrations (see Chapter 1, section 1.2.7). Activation of the nAChR by physostigmine was not blocked by α -btx, in contrast to the physostigmine-competitive antibody FK1 (Okonjo *et al.*, 1991), suggesting that physostigmine was binding to a site distinct from ACh. Mapping of the dominant prototope of the FK1 antibody established that physostigmine bound to the sequence $\alpha 118-137$ of *Torpedo* (Schröder *et al.*, 1994). This region included lysine 125, the amino acid photoaffinity labelled by tritiated physostigmine (Schrattenholz *et al.*, 1993) and overlapped with the cys loop.

The key residue that defines specificity in Cockcroft and colleagues' computer generated model is threonine 133 which is found through $\alpha 1$ to $\alpha 6$ and $\alpha 9$ subunits. However, in $\alpha 7$ and $\alpha 8$, subunits known to bind nicotine, this residue is an arginine. Comparison of insect receptor sequences shows that in D $\alpha 2$ /SAD and $\alpha L1$ it is an arginine whereas in MARA1 and ALS it is a glutamate. If this hypothesis is correct, it begs the question is ALS nicotine sensitive? Although expression of ALS alone has shown no ACh or nicotine evoked responses (Bertrand *et al.*, 1994), co-expression with a chick $\beta 2$ subunit has shown sensitivity to low concentrations of nicotine (EC_{50} : 1.3 μ M). Furthermore, the ALS clone was isolated from a nicotine sensitive strain of *Drosophila*. Therefore, it appears unlikely, that this residue is a candidate amino acid for nicotine insensitivity in *Manduca*. Thus, it seems that MARA1 does not contain any obvious candidate residues pertaining to nicotine insensitivity, suggesting one or more of the following: a) that the

nicotine insensitivity seen in *Manduca* is not due to an altered residue(s) within the agonist binding site; b) that there is a mutated subunit but MARA1 is not it (unlikely as this study presents no evidence of other α -like subunits); or c) that other subunits make contributions to binding in such a way that there is a reduced sensitivity to binding of nicotine. The latter point is the focus of the next chapter, where it will be discussed further.

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-21  MRSVTKYYLH  GVVLFATGCA  GNPDAKRLYD  DLLSNYNKLV    19
    20  RPVLNVSDAL  TVRIKCLKSQ  LIDVNLKNQI  MTTNLWVEQS    59
    60  WYDYKLSWEP  REYGGVEMLH  VPSDHIWRPD  IVLYNNADGN    99
   100  FEVTLATKAT  LNYTGRVEWR  PPAIYKSSCE  IDVEYFPFDQ   139
   140  QTCVMKFGSW  TYDGFQVDLR  HIDEVRGTNV  VELGVDLSEF   179
   180  YTSVEWDILE  VPAVRNEKFY  TCCDEPYLDI  TFNITMRRK    218

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Figure 2.38. Residues in the extracellular region of MARA1, potentially involved in ligand binding. Bold, italicised residues in *Torpedo* were labelled by DDF (Dennis *et al.*, 1986, 1988; Galzi *et al.*, 1990); bold underlined residues mutated to phenylalanine produced decrease in binding affinity for ACh and nicotine in the chick $\alpha 7$ receptor (Galzi *et al.*, 1991).

The residues involved in binding of the antagonist, α -btx, are somewhat disputed. Being a large molecule (8kd) it is thought that α -btx binding results in a large conformational change of the receptor (McCarthy and Stroud, 1989). Indeed, Mishina *et al.* (1985) demonstrated that mutation of cysteine 128 and cysteine 142 in *Torpedo*, thus abolishing their disulphide linkage and the conformation of the subunit and perhaps intersubunit assembly, resulted in no α -btx binding. Furthermore, removal of the putative glycosylation site at asparagine 141 in *Torpedo* prevented α -btx binding, showing that the N-glycosylation site was essential for correct conformation of the nAChR.

It is generally agreed that α -btx binds in the region immediately prior to and after the vicinal cysteines. Evidence from experiments using muscle nAChRs from α -btx resistant species such as snake and mongoose (Barchan *et al.*, 1995; Kachalsky *et al.*, 1995) suggests two subsites at the α -btx binding site: an aromatic subsite at *Torpedo* positions 187 and 189 before the vicinal cysteines and a proline subsite at *Torpedo* positions 194 and 197 after the vicinal cysteines. The neuronal nAChRs that bind α -btx ($\alpha 7$ and $\alpha 8$ on alignment, Figure 2.39) have two close proline residues separated by a tyrosine residue, three residues downstream from the vicinal cysteines. Those that do not bind α -btx have

either one or no proline residues in this region. Kachalsky *et al.* (1995) proposes that the two spaced proline residues can form a loop at the ligand binding site which could be important for binding of α -btx. The proline subsite appears to be critical for α -btx binding whilst the aromatic subsite appears to determine the degree of binding or resistance to α -btx.

Comparison with insect α -like subunits creates an even more confusing picture, as shown in the alignment of this region, Figure 2.39.

| | |
|---------------------------------|---------------|
| | 187 |
| <i>Drosophila</i> SAD | EKYYPCCAE.PYP |
| <i>Schistocerca</i> α L1 | EKYYPCCAE.PYP |
| <i>Drosophila</i> ALS | EKFYSCCEE.PYL |
| <i>Manduca</i> MARA1 | EKFYTCCDE.PYL |
| Chick α 7 | ESFYECCKE.PYP |
| Chick α 8 | ELYECCKE.PYP |
| Chick α 1 | WVYYACCPDTPYL |
| Chick α 2 | SKKYDCCTE.IYP |

Figure 2.39. Alignment of potential α -btx binding site. *Drosophila* SAD: Sawruk *et al.*, 1990a; *Schistocerca* α L1: Marshall *et al.*, 1990; *Drosophila* ALS: Bossy *et al.*, 1988; Chick α 7: Couturier *et al.*, 1990b; Schoepfer *et al.*, 1990; Chick α 8: Schoepfer *et al.*, 1990; Chick α 1, α 2: Nef *et al.*, 1988.

As expected, the neuronal α -btx binding subunits α 7 and α 8 possess the proline subsite, albeit downstream from that for muscle α 1 (muscle at positions 194 and 197) whereas the neuronal non- α -btx binding α 2 does not. Both D α 2/SAD and α L1 have the proline subsite suggesting that they can bind α -btx whereas ALS and MARA1 only possess one proline residue implying that they should be α -btx resistant. Excepting α 1 (which has two) and α 2 (which has none), all subunits have one aromatic residue in the aromatic subsite.

Contradicting this hypothesis is the evidence that bacterial fusion proteins containing the potential ligand binding site of ALS were shown to bind iodinated α -btx with a K_d of 1.7 μ M (Ohana and Gershoni, 1990) whereas α -btx failed to block nicotine-evoked currents in *Xenopus* oocytes expressing D α 2/SAD (Sawruk *et al.*, 1990a). However, this failure of α -btx to block currents was attributed by the authors to almost instantaneous dissociation of the α -btx in the presence of excess competing ligand. On the other hand, combining each of these subunits with chick β 2 subunit (see Homomeric versus

heteromeric receptors; Bertrand *et al.*, 1994) substantiated the experimental data obtained from Ohana and Gershoni (1990) and Sawruk *et al.* (1990a). To confuse matters further, nicotine-evoked currents of α L1 were blocked by α -btx (Marshall *et al.*, 1990; Amar *et al.*, 1995).

It is therefore apparent that the residues used in binding of α -btx to insect subunits cannot be surmised by simple comparison with vertebrates. It is likely that the tertiary and quaternary structures play a large role in the binding of this toxin (Chatrenet *et al.*, 1990).

In order to determine the ability of nAChRs to bind nicotine in *Manduca*, an investigation comparing the binding affinities of various ligands was conducted. This is discussed in Chapter 3.

CHAPTER 3

Pharmacological characterisation of the nAChR from *Manduca sexta*

3.1 Introduction

Recent developments in molecular biology techniques have led to the cloning of numerous nAChR subunits, with similar but distinct sequences (see Chapter 1, section 1.2.4). Consequently, the need to distinguish between receptor subtypes in the nervous system has never been so great. Fortunately, this has been aided by the utilisation of specific ligands (generally natural toxins) which interact with the different receptor subtypes to give a range of binding affinities and selectivities giving a pharmacological profile typical for each receptor subtype. Functional expression, such as that using two electrode voltage clamp recording from receptors expressed in *Xenopus* oocytes aims to impart a particular pharmacological characterisation for each individual receptor subtype (see Chapter 1, section 1.2.5). However, this is far more difficult in the intact nervous system, given the potential for heterogeneity and the possibility that the subunit combinations that are being expressed in the oocytes are not representative of the native nAChR.

3.1.1 *The relationship between radiolabelled binding sites and cloned subunits*

Targeting of particular receptors in intact nervous tissue has generally employed specific radioligands. For nAChRs to be radiolabelled by a cholinergic ligand, they must be able to bind the same unlabelled ligand with high affinity.

[³H]Nicotine

Nicotine, by definition interacts with all nAChRs but only those that bind nicotine with nanomolar affinity are labelled with [³H]nicotine. This property has been used to great advantage in numerous studies of the vertebrate brain nAChRs which have shown that [³H](-)nicotine binds to a single binding site that is both saturable and labelled with high affinity (Marks *et al.*, 1986; Lippiello *et al.*, 1987; Martino-Barrows and Kellar, 1987). Furthermore, the binding site shows 30 to 50-fold selectivity for the (-) stereoisomer compared with the (+) stereoisomer (Martino-Barrows and Kellar, 1987). Despite the potential for diversity, the radiolabelled agonists [³H]nicotine, [³H]acetylcholine, N-[³H]methylcarbamylocholine and [³H]cytisine have virtually identical pharmacological characteristics and regional distribution in rat brain (Marks *et al.*, 1986; Martino-Barrows and Kellar, 1987; Anderson and Americ, 1994), suggesting that they all label the same neuronal nAChR subtype.

Immunopurification of nAChRs from rat brain using a monoclonal antibody (MAb) directed against the chick $\beta 2$ subunit, resulted in the isolation of two subunits, namely $\alpha 4$

and $\beta 2$ (Whiting and Lindstrom, 1987b; see Chapter 1, section 1.2.5). Immunoprecipitation of detergent solubilised rat brain extracts using MAbs specific for either of these subunits removed over 90% of the high affinity [^3H]nicotine binding sites from solution, implying that the [^3H]nicotine binding site was found on receptors comprising $\alpha 4$ and $\beta 2$ subunits (Whiting and Lindstrom, 1986b). This was confirmed by Flores *et al.* (1992) who demonstrated that the high affinity [^3H]cytisine binding sites in rat brain extracts were removed by antisera to $\alpha 4$ and $\beta 2$ subunits (reviewed in Sargent, 1993).

Significantly, the newly marketed radioligand [^3H](\pm)epibatidine putatively binds to $\alpha 3$ subunits (McKay *et al.*, 1994; Houghtling *et al.*, 1995) as well as $\alpha 4$ subunits because of its higher affinity and thus widens the scope of pharmacological investigation for neuronal subunits.

[^{125}I] α -Bungarotoxin

In contrast, the radioligand [^{125}I] α -bungarotoxin ([^{125}I] α -btx) does not appear to bind to the same site (Clarke *et al.*, 1985), preferring the muscle nAChR and certain α -btx sensitive neuronal nAChRs. Indeed, for some time, it was considered that only the muscle type nAChR bound α -btx, such that these neuronal sites were termed α -Bungarotoxin Binding Proteins (α -BgtBPs) 1 and 2 (Schoepfer *et al.*, 1990).

However, the available evidence strongly suggests that these neuronal α -btx binding sites correlate with $\alpha 7$ subunits in chick and possibly also $\alpha 8$ subunits in chick. Couturier *et al.* (1990b) demonstrated that a bacterial fusion protein incorporating the predicted agonist and toxin binding sites of the $\alpha 7$ subunit bound [^{125}I] α -btx and Schoepfer *et al.* (1990) showed that MAbs against recombinant peptide fragments from variable regions of chick $\alpha 7$ and $\alpha 8$ identified bands of affinity purified α -BgtBP on Western blots. Moreover, it was demonstrated that nAChRs containing $\alpha 7$ subunits were the predominant α -BgtBPs in the chick brain as anti- $\alpha 7$ MAbs recognised at least 90% of the α -BgtBPs in depletion and immunoprecipitation assays. Interestingly, anti- $\alpha 8$ MAbs recognised ~15% of the [^{125}I] α -BgtBPs in depletion assays, all of which were recognised by anti- $\alpha 7$ MAbs, suggesting that two different α subunits could be present in the same nAChR (reviewed in Clarke, 1992; see Chapter 1, section 1.2.5). More recently, Elgoyhen *et al.* (1994) have demonstrated that the $\alpha 9$ subunit displays high affinity for α -btx, however as this was not located in the central nervous system (see section 3.1.2, mixed nAChRs) it is unlikely that it accounts for any remaining α -BgtBPs.

3.1.2 AChRs in insects

Nicotinic AChRs

As discussed in Chapter 1 (sections 1.1.2 and 1.3.1), radioligand binding on the nervous system tissues of a variety of insects has demonstrated the existence of an α -btx binding component that exhibits the properties of an AChR. Evidence of an α -btx binding site in *Manduca* was first presented by Sanes *et al.* (1977) who showed that α -btx binding activity increased throughout metamorphosis, was specific to nervous tissue and was blocked by the cholinergic agents carbamylcholine, d-tubocurarine and nicotine. Its pharmacological profile was such as to be considered an AChR. This was supported by further evidence demonstrating that the α -btx binding sites were localised in the glomeruli of the antennal lobes which contain all of the ultrastructurally known synapses (Hildebrand *et al.*, 1979; see Chapter 1, section 1.1.2). Binding studies performed using *Drosophila melanogaster* (Schmidt-Nielsen *et al.*, 1977; Dudai, 1978; Rudloff, 1978; Schloss *et al.*, 1988) and *Musca domestica* (Mansour *et al.*, 1980; Cattell *et al.*, 1980a) further defined this α -btx binding component as a nicotinic AChR with pharmacological characteristics close to those found in vertebrates. It is tempting to draw correlations between vertebrate and insect pharmacology. Given that in vertebrates, $\alpha 7$ subunit-containing receptors bind α -btx, it could be very tentatively suggested that an $\alpha 7$ -like subunit nAChR exists in insects. As yet, various interpretations of molecular and pharmacological data remain inconclusive.

Mixed AChRs

There is little evidence in the literature to suggest that an equivalent site to the vertebrate [3 H]nicotine / [3 H]acetylcholine site exists in insects. Instead, a putative mixed AChR displaying both nicotinic and muscarinic pharmacological characteristics has been proposed. Initially, Eldefrawi and O'Brien (1970) discovered that binding of [3 H]muscarone (an AChR agonist effective on both nicotinic and muscarinic synapses) in *Musca* was inhibited by both nicotinic and muscarinic ligands. Since then, this putative mixed receptor has been purified and extensively characterised using the radiolabels [3 H]acetylcholine, [3 H]decamethonium and [3 H]nicotine (Cattell *et al.*, 1980b; Mansour *et al.*, 1980). This receptor does not bind α -btx but nicotine and muscarine inhibit, for example, [3 H]decamethonium binding with very similar IC_{50} values (6.5 μ M and 4 μ M respectively). Furthermore, this putative receptor can be solubilised without the use of detergents and is 50-fold and 1000-fold more concentrated than the nicotinic and muscarinic receptors of *Musca* respectively (Eldefrawi *et al.*, 1982). Given that there is no functional electrophysiological data from *Musca* neurons, proving the existence of a

mixed receptor, there is some speculation over the origin and physiological role of these mixed receptors in *Musca*. However, a similar putative receptor has been isolated from nerve cord extracts of the cockroaches, *Gromphadorhina portentosa* and *Periplaneta americana* (Aziz and Eldefrawi, 1973). More recently, a vertebrate subunit designated $\alpha 9$, has been identified that also has unusual mixed nicotinic-muscarinic pharmacology (Elgoyhen *et al.*, 1994). When the $\alpha 9$ subunit was expressed either alone or in conjunction with $\beta 2$ or $\beta 4$ subunits in *Xenopus* oocytes it was not activated by either nicotine or muscarine, the definitive agonists of the nAChR and mAChR respectively. However, currents were elicited from $\alpha 9$ injected oocytes by the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) and the muscarinic agonist oxotremorine-M. *In situ* hybridisation demonstrated that $\alpha 9$ subunits were not found in the central nervous system, rather they were located in the pars tuberalis of the hypophysis, the nasal epithelium, the hair cells of the cochlea and the skeletal muscle of the tongue. How this finding reflects on the insect mixed receptor is unclear.

Muscarinic AChRs

Muscarinic AChRs have also been found in membrane extracts of nervous tissue from insects using the radiolabel [^3H]quinuclidinyl benzilate ([^3H]QNB). Unlike vertebrates, in insects, muscarinic receptors appear to be fewer in quantity than nicotinic receptors (B_{max} of 65fmol/mg protein for [^3H]QNB binding compared with a B_{max} of 300-800fmol/mg protein for [^{125}I] α -btx binding in *Drosophila*; Dudai, 1978; Haim *et al.*, 1979). Two putative mAChRs in *Manduca* have been recently characterised using the inhibition of [^3H]QNB binding. The high affinity site has a B_{max} of 23fmol/mg protein whereas the low affinity site has a B_{max} of ~800fmol/mg protein (Trimmer *et al.*, 1993).

Manduca AChRs

Interestingly, Sanes *et al.* (1977) suggested that the α -btx binding component investigated in their study of *Manduca* was of the mixed nicotinic-muscarinic subtype, as it was sensitive to both nicotine and atropine. However, full dose response curves were not obtained, the one concentration of competing ligand was high (200 μM) and a crude membrane preparation was used. As it was shown in *Musca* that the mixed receptor did not bind α -btx (see mixed AChRs above) it seems unlikely that this is the case in *Manduca*. This is further discussed in section 3.5.

Functional analysis of putative receptors on the motoneurone PPR of *Manduca* suggested the presence of both nicotinic and muscarinic AChRs (see Chapter 1, section 1.1.3; Trimmer and Weeks, 1989). As with an earlier study completed by Morris (1984),

it was suggested that the nAChRs tested were relatively insensitive to nicotine and that this may be due to a modified nAChR in *Manduca* (see Chapters 1 and 4 for a more extensive discussion).

Therefore the aim of this study (Chapter 3) is to provide a pharmacological profile of the nAChR in *Manduca* by employing the specific binding of the radioligands [125 I] α -btx and [3 H](\pm)epibatidine and their inhibition by assorted ligands. Most importantly, the endogenous ligand ACh and the definitive ligand nicotine are investigated. In addition, another alkaloid found in tobacco, anabasine, a cholinergic insecticide, imidacloprid and the highly potent frog toxin epibatidine are used. α -Btx and methyllycaconitine (MLA), a selective $\alpha 7$ inhibitor, are included as the antagonists in the study. The effects of muscarinic agents and acetylcholinesterase (AChE) inhibitors are also determined. Comparison of adults and larvae is made as it would be expected that larvae would show greater discrimination for nicotine binding than adults if Trimmer and Weeks' (1989) observations hold for binding experiments (see Chapter 1, section 1.1.3).

3.1.3 Characteristics of specific binding

In a radioligand binding assay, two types of binding site are available to which the radioligand can bind. These are specific sites and non-specific sites. In the case of a selective and highly potent ligand such as [125 I] α -btx on α -btx sensitive nAChRs, most of the binding will be to specific sites. However, the following criteria (Satelle, 1985) must be met in order to conclude that specific rather than non-specific binding is taking place:

- 1) The specific binding should saturate with increasing concentrations of radiolabel, showing that there is a finite number of receptor sites.
- 2) Ligands with the same receptor binding site specificity as the radiolabel should displace binding of the radiolabel at pharmacologically effective concentrations whilst those ligands that have a different receptor binding site specificity should be ineffective.
- 3) The saturating, specific binding should be located at tissue regions known to contain the receptors.

Although not a criterion, it is also necessary to select conditions whereby specific binding increases linearly with increasing tissue concentration. In this study, points 1 and 2 are studied. Hildebrand *et al.* (1979) have previously addressed point 3. For a detailed

explanation of receptor-ligand interactions refer to Bylund and Yamamura (1990). Binding studies were carried out in collaboration with Robert Lind (University of Bath).

3.2 Materials

Manduca were obtained from the University of Bath *Manduca* colony, where they were reared in a controlled environment room at $25 \pm 0.5^{\circ}\text{C}$, $50 \pm 10\%$ relative humidity, with a light, dark cycle of 17 : 7 hours. Larvae were raised on an artificial diet (Bell and Joachim, 1976) comprising wheatgerm, casein, sucrose, yeast, salts, vitamins and preservatives, bound together with agar in water (J. Gillespie, 1995). *Manduca* were precisely staged and dissected by Professor Stuart Reynolds and Audrey Brown.

α -Btx and (\pm)epibatidine dihydrochloride were supplied by Research Biochemicals International (RBI) and distributed by Semat, St. Albans, U.K. [^{125}I] α -Btx (specific activity: $\sim 750\text{Ci/mmol}$) was prepared by Dr. Adrian Rogers (University of Bath). [^3H](\pm)Epibatidine (specific activity: 57Ci/mmol ; 1mCi/ml) was supplied by NEN-DuPont (U.K.) Ltd., Stevenage, U.K.

ACh bromide, (\pm)anabasine (neonicotine) and (-)-nicotine hydrogen di (+) tartrate, were obtained from the Sigma-Aldrich Chemical Company Ltd. (Sigma), Poole, U.K. Imidacloprid was a gift from Zeneca Agrochemicals, Jealott's Hill Research Station, U.K. MLA citrate was obtained from RBI.

The muscarinic ligands, atropine, (\pm)muscarine chloride and oxotremorine were purchased from Sigma, as were the acetylcholinesterase inhibitors, BW284c51 (1,5 bis(4 allyl dimethylammoniumphenyl)pentan-3-one dibromide), neostigmine bromide, physostigmine (eserine) and THA (9 amino 1,2,3,4 tetrahydroacridine hydrochloride).

The protease inhibitors, leupeptin, pepstatin A and PMSF (phenylmethylsulphonylfluoride) were from Sigma, as were BSA fraction V and PEI (polyethylenimine). GF/C filters were obtained from Whatman International Ltd., Maidstone, U.K. The Bio-Rad protein assay was supplied by Bio-Rad Laboratories Ltd., Hemel Hempstead, U.K. All other chemicals were from Fisons Scientific Equipment, Loughborough, U.K.

All drugs used in binding assays were made as 10mM stock solutions in incubation buffer except MLA (in 100% ethanol) and imidacloprid (in acetone) and stored in aliquots at -20°C .

3.3 Methods

3.3.1 Membrane preparation

The protocols used were based on the *Drosophila* particulate membrane preparation of Schmidt-Nielsen *et al.* (1977).

Preparation of adult P2 membranes

Manduca pharate adults were killed by decapitation. Brains (protocerebrum and optic lobes) were dissected and either used immediately or frozen in liquid nitrogen and stored at -70°C.

Solutions were kept on ice throughout and all equipment was cooled to 4°C before use. Generally, adult preparations used approximately 250 brains. Brains were homogenised manually in an homogeniser (glass mortar, PTFE pestle) containing (7%(w/v)) isolation buffer (0.32M sucrose, 100µM EDTA, pH7.2) supplemented with the protease inhibitors leupeptin (1µM), pepstatin A (1µM) and PMSF (200µM). The homogenate was centrifuged at low speed for 15min and the resulting supernatant (S1) split between microcentrifuge tubes and centrifuged at 15000g for 30min. Pellets (P2) were combined and resuspended in incubation buffer (0.05M Tris-Cl, 0.12M NaCl, 100µM EDTA, 0.25% (w/v) BSA, pH7.4) to a concentration of 10 brains/ml. The P2 membrane preparation was stored in aliquots (1ml) at -20°C.

Preparation of larval P2 membranes

First instar larvae were collected within one day of hatching from eggs, frozen in liquid nitrogen and stored at -70°C until required.

Solutions were kept on ice throughout and all equipment was cooled to 4°C before use. Whole larvae (approximately 10g per preparation) were homogenised in a rotary homogeniser (glass mortar, PTFE pestle) by repeated up and down strokes at approximately 3000rpm, using supplemented isolation buffer (66% (w/v)) as for adults. The homogenate was centrifuged at low speed for 15min. The resulting supernatant (S1) was filtered through nylon cloth and the filtrate split into microtubes and centrifuged as for adults. Pellets (P2) were combined and resuspended in incubation buffer to a concentration of 0.8g larvae/ml. P2 pellets were stored in 1ml aliquots at -20°C.

3.3.2 Protein determination

The concentration of protein in each preparation was measured using the Bio-Rad Protein Assay based on the Bradford dye binding procedure (Bradford, 1976). Standard curves were constructed using BSA over the range 5-20µg/ml. Duplicates (total volume: 800µl) of adult or larval P2 membranes in incubation buffer were serially diluted in water between the range 1:200 to 1:3200. Dye reagent concentrate (200µl) was added to each sample, mixed thoroughly and the colour left to develop over 10min. Water controls, treated with dye reagent concentrate were used as zero absorbance blanks. The absorbancies at 595nm were measured on a Perkin Elmer UV/VIS Lambda 11 spectrometer. The BSA standard curves obtained were used to determine protein concentrations of the membrane preparations, taking into account the BSA (2.5mg/ml) contained in the incubation buffer.

3.3.3 [¹²⁵I]α-Bungarotoxin GF/C filter binding assays

The following experiments were based on these three principles behind the experimental measure of specific binding.

- 1) Total binding of radioligand to all available sites was determined by incubation of membranes with radioligand.
- 2) Non-specific binding of the radioligand was determined by displacement of total binding by excess unlabelled ligand that competes for the same binding site.
- 3) Specific binding of the radioligand was calculated as the difference between total and non-specific binding.

The protocol followed was based on that by Schmidt-Nielsen *et al.* (1977). Assays were carried out as duplicate determinations at room temperature, using incubation buffer (as previously) and P2 membranes (23µg for adults; 93µg for larvae) in a total volume of 200µl. Total binding was determined by the addition of [¹²⁵I]α-btx to a final concentration of 1nM in adult assays or 5nM in larval assays and non-specific binding was determined in the presence of excess unlabelled α-btx (final concentration of 1µM for adults or 5µM for larvae). Controls without P2 membranes were also included. Samples were mixed thoroughly and incubated at room temperature for 60min.

Incubation buffer (3ml) was added to each sample and bound and free ligand separated by rapid filtration through GF/C filters (pre-soaked overnight in 0.3% (v/v) PEI) on a Millipore vacuum manifold. Sample tubes were rinsed once in incubation buffer and the filters washed twice with 5ml incubation buffer within 30s. Bound radiolabel on the filters

was counted on a Packard Cobra II gamma counter. Specific binding was calculated by subtracting non-specific binding from total binding. To ascertain total levels of radioactivity, a count of [125 I] α -btx corresponding to the total amount of radioligand used in an individual sample was taken for each experiment.

3.3.3.1 Protein dependence of [125 I] α -bungarotoxin binding

The dependence of [125 I] α -btx binding on protein concentration was investigated for both adult and larval P2 membranes. P2 membranes were diluted in incubation buffer to give different protein concentrations and total and non-specific binding determined as above. Specific binding was calculated as the difference between total and non-specific binding.

3.3.3.2 Time and temperature dependence of [125 I] α -bungarotoxin binding

To establish appropriate reaction conditions, the rate of [125 I] α -btx binding to P2 membranes was measured for adults over 3h, at room temperature (25°C) and at 37°C. Specific binding was determined for each time point as the difference in total and non-specific binding as described above.

3.3.3.3 Saturation binding

The saturability of [125 I] α -btx binding sites on P2 membranes from both adults and larvae was investigated. Concentration ranges for [125 I] α -btx of 0.1-30nM and 0.5-30nM were used for adults and larvae P2 membranes respectively. Total and non-specific binding was determined for each [125 I] α -btx concentration as described above and the resultant specific binding isotherm used to derive B_{max} and K_d values (see section 3.3.5).

3.3.3.4 [125 I] α -Bungarotoxin competition binding assays

A variety of cholinergic ligands were used in competition binding assays, to adult and larval P2 membranes. Drugs, over an appropriate concentration range, generally nM to mM, were pre-incubated with P2 membranes in incubation buffer, at room temperature for 10min, before addition of [125 I] α -btx, and incubation of samples as described above. Total and non-specific binding were determined as above. The resultant specific binding was used to calculate the IC_{50} and K_i value for each drug (see section 3.3.5).

Pre-incubation of (-)nicotine

To detect the existence of substantial nicotine breakdown by metabolic enzymes in the P2 membrane preparations, the first (-)nicotine competition binding assay with [¹²⁵I]α-btx was carried out using pre-incubation times of 0, 10 and 60min. The zero time point was incubated at 4°C, whereas 10 and 60min pre-incubation samples were incubated at room temperature. Specific binding was determined as the difference in total and non-specific binding as described above.

3.3.4 [³H](±)Epibatidine binding assays

[³H](±)Epibatidine binding assays were carried out as duplicate determinations at room temperature, using incubation buffer (as previously) and P2 membranes (23μg for adults; 93μg for larvae) in a total volume of 200μl. Total binding was determined by the addition of [³H](±)epibatidine to a final concentration of 1 or 10nM while α-btx and (-) nicotine were used at high concentrations to assess specific [³H](±)epibatidine binding to adult and larval membranes. α-Btx (1μM for adults, 5μM for larvae) and (-)nicotine (1mM for adults and larvae) were pre-incubated with P2 membranes in incubation buffer, at room temperature for 10min, prior to addition of 1 or 10nM [³H](±)epibatidine and incubation of samples. Controls without P2 membranes were also included. Samples were mixed thoroughly and incubated at room temperature for 60min. Bound radioligand on the filters was counted in 5ml Optiphase 'Safe' scintillant using a Packard Tricarb 1600 TR scintillation spectrometer (counting efficiency 40%). Controls without membrane and total radioactivity counts were taken.

3.3.5 Data analysis

The parameters B_{max} (the maximum number of binding sites) and K_d (the dissociation constant for the equilibrium binding of the radiolabel) were determined from [¹²⁵I]α-btx saturation binding. Saturation data and also protein dependence and time course assay data were fitted to first order linear regression plots or rectangular hyperbolas using equation (1):

$$y = (B_{\max} \cdot x) / (K_d + x) \quad (1)$$

where y = amount of radioligand bound and x = protein or radioligand concentration. B_{max} and K_d were derived from the curve fit of the specific binding isotherm.

Saturation data was transformed using equation (2):

$$B/F = (-1/K_d)B + B_{\max}/K_d \quad (2)$$

where B = bound radiolabel and F = free radiolabel, to produce a Scatchard plot (bound/free radiolabel versus bound radiolabel) indicative of a single or multiple binding site(s) model.

The parameters, IC_{50} (the concentration of the competing drug that inhibits 50% of the specific binding) and K_i (the affinity of the inhibitor for the receptor) were determined from competition binding assays. IC_{50} values were derived for each experiment by fitting to the Hill equation (3):

$$B = 100\% / (1 + ([\text{ligand}] / IC_{50})^{nH}) \quad (3)$$

where B = % bound radiolabel, [ligand] = concentration of displacing ligand and nH = Hill number.

Each IC_{50} value was used to calculate the K_i value by the method of Cheng and Prusoff (1973) (4):

$$K_i = IC_{50} / (1 + ([\text{radioligand}] / K_d)) \quad (4)$$

where [radioligand] = concentration of radioligand.

For each concentration of drug, the mean \pm range from two or mean \pm sem from at least three experiments were plotted and fitted to a single binding site model, using the non-linear least squares fitting facility of the SigmaPlot for Windows Version 2.0 graphics program.

3.4 Results

3.4.1 Optimisation of [125 I] α -bungarotoxin binding

Preliminary experiments were carried out on [125 I] α -btx binding to membrane preparations in order to establish appropriate reaction conditions.

Membrane preparations

Initial preparations of adult membranes demonstrated that dissected brain tissue resulted in a more purified preparation than dissected whole head tissue. Consequently, brains of adults were used for binding assays. Conversely, dissections of larval fifth instar nerve cords proved exceedingly time consuming and resulted in such little tissue that binding assays had to be performed on whole first instar larvae.

Comparison of [125 I] α -btx binding assays on both fresh and frozen brain tissue indicated that the freezing process did not impair binding; this allowed for large membrane preparations from the collection of adult and larval tissue. Binding to the S2 supernatant in comparison with the P2 pellet established that the pellet gave a greater difference between specific [125 I] α -btx binding and non-specific [125 I] α -btx binding than the supernatant.

[125 I] α -Bungarotoxin binding

Initial assays were carried out as triplicate determinations, however, due to the paucity of tissue these were later performed as duplicate determinations.

Separation of bound and free radiolabel was completed as quickly as possible in order to prevent dissociation of the receptor-radioligand complex. Non-specific binding to membranes and GF/C filters was minimised by washing with copious amounts of incubation buffer containing BSA. In addition, GF/C filters were pre-treated with PEI to reduce non-specific binding (Bruns *et al.*, 1983).

Protein dependence of [125 I] α -bungarotoxin binding

The protein dependence of [125 I] α -btx binding was determined in order to select a protein concentration which gave both a good signal to noise ratio and was proportional to the specific [125 I] α -btx binding (i.e. on the linear part of the curve). The effect of altering the protein concentration and thus receptor concentration on the binding of [125 I] α -btx varied between adults and larvae, as shown in Figure 3.1. For both adults and larvae, total binding increased linearly with increasing protein concentration. Non-specific binding to

adult membranes was very low (reaching approximately 3000cpm at 45 μ g/assay compared with approximately 26000cpm for total binding at the same protein concentration, in the example shown), demonstrating only a minor linear increase with increasing protein concentration. In contrast, non-specific binding to larval membranes was very high - being close to 50% of the total counts bound and increased steeply with increasing protein concentration. Consequently, this resulted in a different profile for the specific binding for each type of membrane preparation. Specific binding for adults emulated total binding, showing a steep linear increase with increasing protein concentration. Specific and non-specific binding could be distinguished easily at a protein concentration of 23 μ g/assay and accordingly this protein concentration was selected for subsequent binding assays. Specific binding for larvae however, was linear over the range 0-100 μ g protein/assay and then levelled off. At ~150 μ g protein/assay specific binding counts were exceeded by those for non-specific binding. Hence, a protein concentration of 93 μ g/assay was selected for subsequent binding assays.

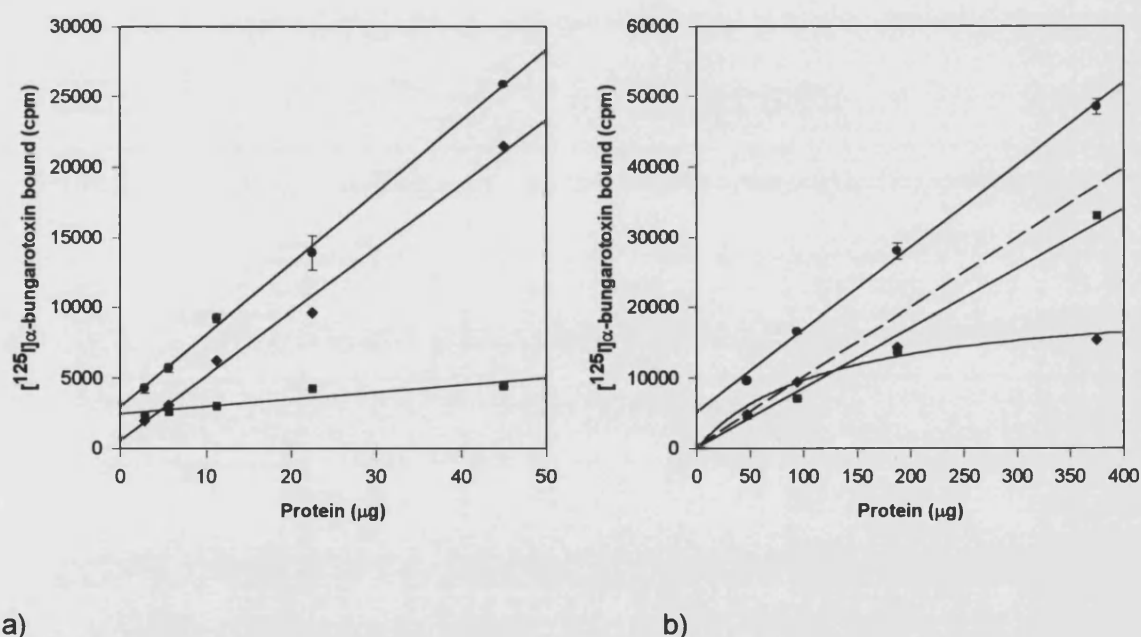


Figure 3.1. Protein dependence of $[^{125}\text{I}]\alpha\text{-btx}$ binding to P2 membranes. $[^{125}\text{I}]\alpha\text{-Btx}$ was incubated for 1h with increasing protein concentration in the presence and absence of unlabelled $\alpha\text{-btx}$ as described in section 3.3.3.1. Bound and free $[^{125}\text{I}]\alpha\text{-btx}$ were separated by vacuum filtration on GF/C filters. a) Data from adult P2 membranes. b) Data from larval P2 membranes. Points represent mean \pm range for duplicate counts of one experiment. Total $[^{125}\text{I}]\alpha\text{-btx}$ bound (●) and non-specific $[^{125}\text{I}]\alpha\text{-btx}$ bound (■) were used to calculate specific $[^{125}\text{I}]\alpha\text{-btx}$ bound (◆).

Time and temperature dependence of [¹²⁵I]α-bungarotoxin binding

The effect of incubation time and temperature on [¹²⁵I]α-btx binding to adult membranes, (Figure 3.2), was investigated to show that the reaction had reached a steady state. Incubation at room temperature (25°C) and 37°C revealed almost identical binding curves, indicating that incubation at room temperature was an appropriate choice for subsequent assays. An approximate T_{1/2} was derived from the rectangular hyperbolic curve fit of specific [¹²⁵I]α-btx binding: at room temperature T_{1/2} was 11min whereas at 37°C T_{1/2} was 13min. Steady state was reached within 60min. In contrast, previous experiments on *Manduca* membranes had demonstrated that the α-btx binding reaction took 16h to reach steady state (Sanes *et al.*, 1977).

3.4.2 Saturation binding of [¹²⁵I]α-bungarotoxin binding

The effect of increasing the [¹²⁵I]α-btx concentration on specific binding to P2 membranes from both adults and larvae is shown in Figure 3.3.

Total and specific binding data were fitted to a rectangular hyperbola whilst non-specific binding data was fitted to a first order linear regression. For adults, subtraction of linear non-specific binding from saturating total binding resulted in specific binding that was almost completely saturated at 30nM. Scatchard analysis of the specific binding (inset) was linear suggesting a single α-btx binding site. From the curve fit, the apparent B_{max} was 815fmol/mg protein and the apparent K_d was 6.5nM (Table 3.1). For larvae, the difference between very high linear non-specific binding and only slightly higher total binding resulted in low specific binding that was saturating at 15nM. A linear Scatchard plot of the specific binding (inset) was obtained suggesting a single class of α-btx binding site. Curve fit data for the experiment shown, gave an apparent B_{max} of 138fmol/mg protein and an apparent K_d of 7.4nM (Table 3.1).

| | B _{max} (fmol/mg protein) | K _d (nM) |
|---------------------|------------------------------------|---------------------|
| Adults [•] | 815 | 6.5 |
| Larvae [■] | 119 ± 19 | 7.6 ± 0.2 |

Table 3.1. Parameters for saturation binding from adult and larval P2 membranes. [•]adults, data are for one experiment performed in duplicate; [■]larvae, data are the mean ± range of two experiments performed in duplicate.

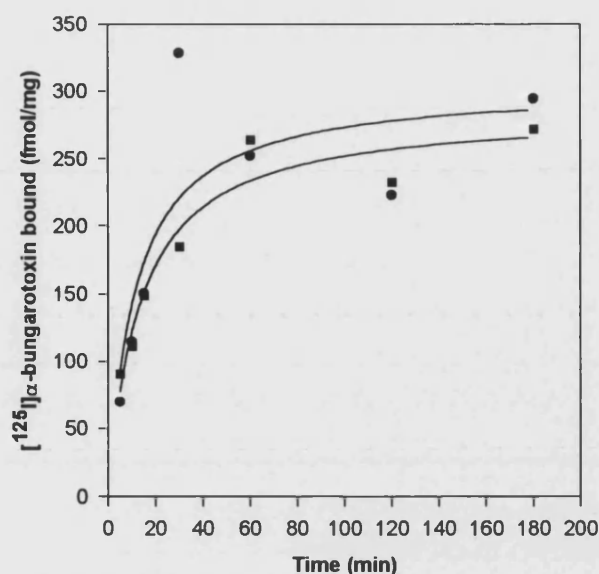
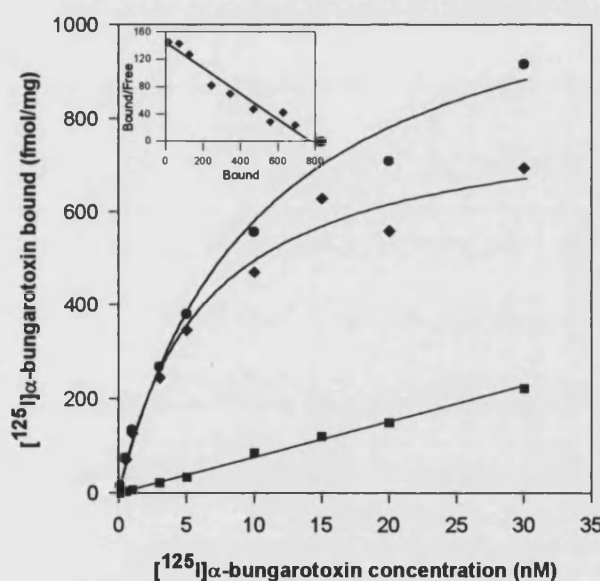
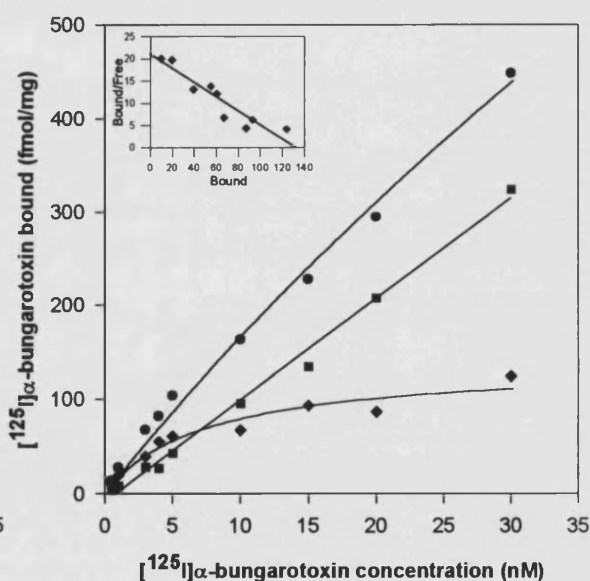


Figure 3.2. Time and temperature dependence of $[^{125}\text{I}]\alpha\text{-btx}$ binding. Adult P2 membranes were incubated with $[^{125}\text{I}]\alpha\text{-btx}$ at room temperature (●) and at 37°C (■) and for increasing time periods in the presence and absence of excess unlabelled $\alpha\text{-btx}$, as described in section 3.3.3.2. Bound and free $[^{125}\text{I}]\alpha\text{-btx}$ were separated by vacuum filtration on GF/C filters. Specific $[^{125}\text{I}]\alpha\text{-btx}$ binding was fitted to a rectangular hyperbola.



a)



b)

Figure 3.3. Saturable $[^{125}\text{I}]\alpha\text{-btx}$ binding to P2 membranes from a) adults and b) larvae. P2 membranes were incubated with increasing concentrations of $[^{125}\text{I}]\alpha\text{-btx}$ for 1h in the presence and absence of unlabelled $\alpha\text{-btx}$, as described in section 3.3.3.3. Bound and free $[^{125}\text{I}]\alpha\text{-btx}$ were separated by vacuum filtration on GF/C filters. Specific $[^{125}\text{I}]\alpha\text{-btx}$ binding data was fitted to a rectangular hyperbola and used to calculate the maximum number of binding sites, B_{max} and the dissociation constant, K_d . Scatchard plots (insets) show linearity, indicating one class of $[^{125}\text{I}]\alpha\text{-btx}$ binding site.

3.4.3 [125 I] α -Bungarotoxin competition binding

The effect of pre-incubation of P2 membranes with a variety of unlabelled cholinergic ligands was investigated in order to assess the different ligands for their potencies at the [125 I] α -btx binding site. Unlabelled α -btx completely displaced [125 I] α -btx with high affinity in both adult and larval membranes (Figure 3.4). The data were used to generate IC₅₀ values, which were converted to K_i values using the Cheng-Prusoff equation (see section 3.3.5). Mean data were fitted to the Hill equation. Displacement of a radioligand by the same unlabelled ligand should result in identical K_d and K_i values, as under these conditions they are the same measurement. For adult membranes, a K_i value of 1.3nM was close to the K_d value of 6.5nM. For larval membranes, a slightly lower affinity giving a K_i value of 9.7nM was also close to the K_d value of 7.6nM (see Table 3.1). The Hill numbers of ~0.9 and ~0.7 for adults and larvae respectively (see Table 3.6), were close to unity, indicating a single binding site consistent with the saturation data.

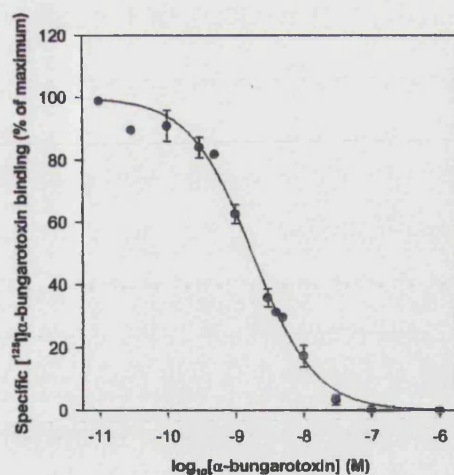
Assessment of (-)nicotine pre-incubation time on the inhibition of [125 I] α -bungarotoxin

(-)Nicotine was pre-incubated with adult membranes at times of 10 and 60min and compared with displacement using no pre-incubation, to assess for nicotine breakdown by putative metabolic enzymes present in the CNS of *Manduca* (Morris, 1983a). Figure 3.5 shows data for one experiment at each of these three time points. K_i values were calculated for each set of data and are shown in Table 3.2.

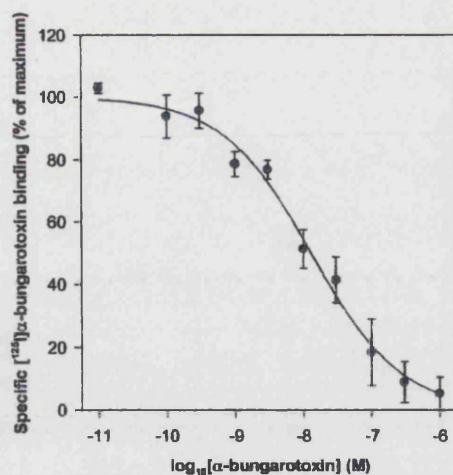
| | 0min, 4°C | 10min, rt | 60min, rt |
|---------------------------|-----------|-----------|-----------|
| K _i (μ M) | 0.75 | 1.05 | 2.80 |

Table 3.2. Effect of nicotine pre-incubation time on the displacement of [125 I] α -btx binding as measured by K_i values.

Nicotine was able to displace [125 I] α -btx with micromolar affinity. The K_is were very similar for each incubation, suggesting that under the assay conditions used nicotine was not degraded.



a)



b)

Figure 3.4. Competition binding assay of α -btx at the $[^{125}\text{I}]\alpha$ -btx binding site in a) adults and b) larvae. Increasing concentrations of α -btx (\bullet) were pre-incubated with P2 membranes for 10min before incubation with $[^{125}\text{I}]\alpha$ -btx for 1h, as described in section 3.3.3.4. Total and non-specific $[^{125}\text{I}]\alpha$ -btx binding were determined by the incubation of P2 membranes and $[^{125}\text{I}]\alpha$ -btx binding in the absence and presence of excess unlabelled α -btx respectively. Bound and free $[^{125}\text{I}]\alpha$ -btx were separated by vacuum filtration on GF/C filters. Points represent specific $[^{125}\text{I}]\alpha$ -btx binding for the mean \pm sem for 3 independent assays. IC_{50} values obtained for each set of data were used to calculate the inhibition constant, K_i . Mean data were fitted to the Hill equation.

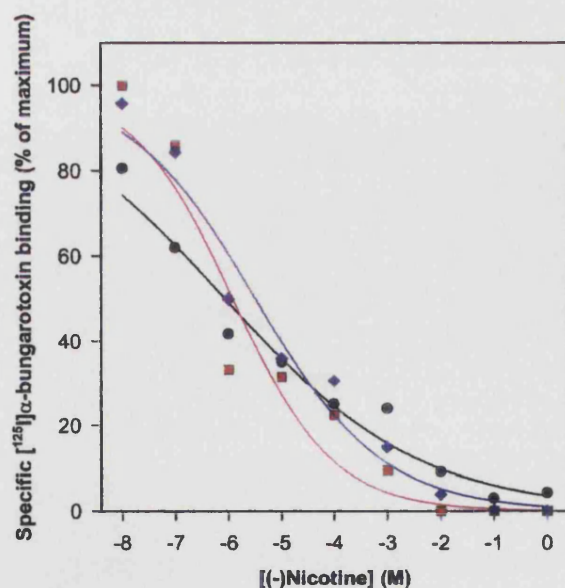


Figure 3.5. Effect of pre-incubation on the inhibition of specific $[^{125}\text{I}]\alpha$ -btx binding by (-)-nicotine. Adult P2 membranes and increasing concentrations of (-)-nicotine were pre-incubated for 0 (\bullet), 10 (\blacksquare) and 60 (\blacklozenge) min before addition of 1nM $[^{125}\text{I}]\alpha$ -btx for 1h, as described in section 3.3.3.4. Total and non-specific binding were determined by the absence and presence of excess unlabelled α -btx respectively. Bound and free $[^{125}\text{I}]\alpha$ -btx were separated by vacuum filtration on GF/C filters. Data were fitted to the Hill equation.

Assessment of inhibition by acetylcholinesterase inhibitors

It was imperative to supplement ACh assays with AChE inhibitors, thus preventing breakdown of ACh from the AChE present in the membrane preparations. A preliminary experiment evaluated several AChE inhibitors for their lack of ability in binding to the α -btx binding site, at a concentration (10 μ M) high enough to stop degradation of ACh. The effect of the AChE inhibitors, BW284c51, neostigmine, physostigmine and THA on displacement of [125 I] α -btx is shown in Figure 3.6. Specific binding (%) of [125 I] α -btx is shown in Table 3.3.

| Acetylcholinesterase inhibitor (10 μ M) | Specific [125 I] α -btx binding (%) |
|---|--|
| BW284c51 | 81.3 |
| Neostigmine | 97.2 |
| Physostigmine | 27.9 |
| THA | 46.1 |

Table 3.3. Inhibition of [125 I] α -btx binding by acetylcholinesterase inhibitors.

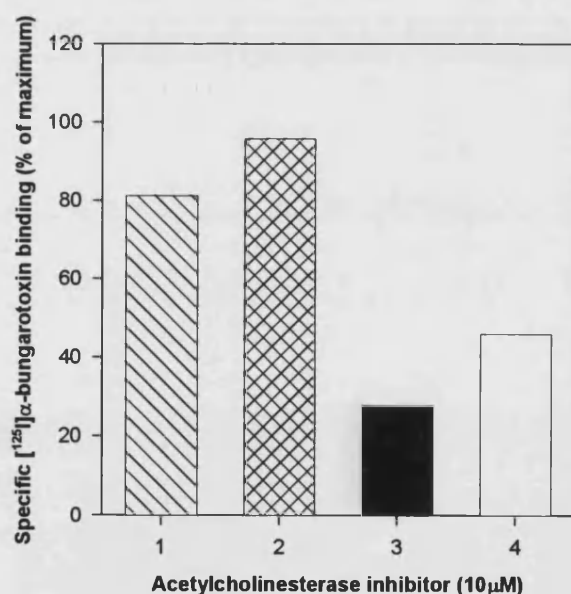


Figure 3.6. Inhibition of specific [125 I] α -btx binding by acetylcholinesterase inhibitors. Adult P2 membranes were pre-incubated with the acetylcholinesterase inhibitors BW284c51 (1), neostigmine (2), physostigmine (3) and THA (4) (all at 10 μ M) for 10min before incubation with [125 I] α -btx for 1h as described in section 3.3.3.4. Total and non-specific binding were determined by incubation of P2 membranes and [125 I] α -btx for 1h in the absence and presence of excess unlabelled α -btx respectively. Bound and free [125 I] α -btx were separated by vacuum filtration on GF/C filters.

At a concentration of 10 μ M (high enough to inhibit AChE in P2 membranes thus preventing ACh breakdown) the rank order of potency was physostigmine > THA > BW284c51 > neostigmine. Thus, neostigmine was used in further ACh competition binding assays to inhibit AChE, as this drug appeared to have the least ability to bind to the [125 I] α -btx binding site.

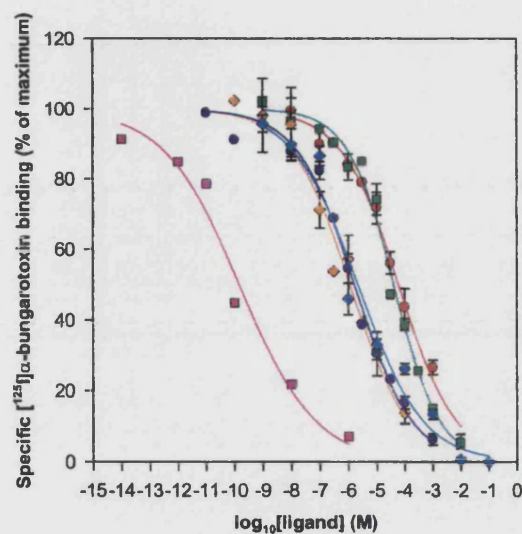
Inhibition by nicotinic ligands

The effects of inhibition of [125 I] α -btx binding by a number of nicotinic ligands is shown in Figure 3.7. The data obtained from adult membranes are much clearer than that from larval membranes, the displacement curves being more defined and the error bars much smaller. This is probably due to the increased purity of the P2 preparation obtained from adults and the resulting low non-specific binding in comparison with that from larvae. Unlabelled ligands did not always result in 100% inhibition of [125 I] α -btx binding (0% specific binding) as the concentrations of unlabelled ligand necessary to reach this inhibition were too high.

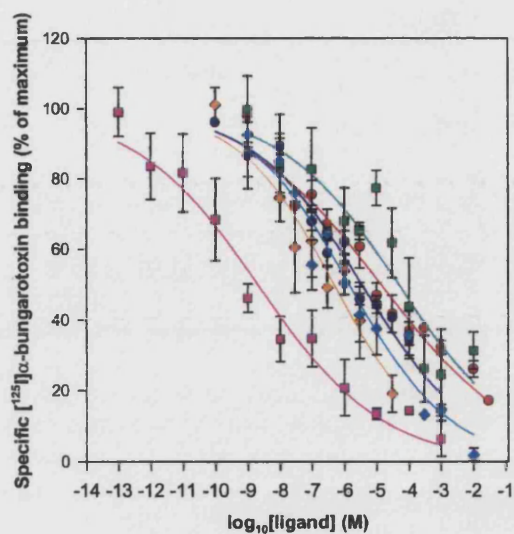
The data were used to generate IC₅₀ values, which, for comparison with other insects, were converted to K_i values using the Cheng-Prusoff equation as in section 3.3.5. α -Btx is also included for direct comparison. The IC₅₀ value, K_i value and potency relative to ACh for each ligand are shown in Table 3.4.

| LIGAND | ADULTS IC ₅₀ (M) | ADULTS * K _i (M) | ADULTS Relative Potency | LARVAE IC ₅₀ (M) | LARVAE * K _i (M) | LARVAE Relative Potency |
|-----------------|---------------------------------------|--------------------------------|-------------------------------|---------------------------------------|--------------------------------|-------------------------------|
| α -Btx | 1.6 \pm 0.2 $\times 10^{-9}$ (3) | 1.3 \pm 0.1 $\times 10^{-9}$ | 43077 | 1.7 \pm 0.7 $\times 10^{-8}$ (3) | 9.7 \pm 3.8 $\times 10^{-9}$ | 824 |
| ACh | 6.4 \pm 1.4 $\times 10^{-5}$ (3) | 5.6 \pm 1.2 $\times 10^{-5}$ | 1 | 1.3 \pm 0.5 $\times 10^{-5}$ (3) | 8.0 \pm 3.2 $\times 10^{-6}$ | 1 |
| (+)-Anabasine | 4.2 \pm 0.4 $\times 10^{-5}$ (3) | 3.6 \pm 0.3 $\times 10^{-5}$ | 1.5 | 2.8 \pm 1.6 $\times 10^{-4}$ (3) | 1.6 \pm 0.9 $\times 10^{-4}$ | 0.05 |
| (+)-Epibatidine | 9.6 \pm 3.4 $\times 10^{-7}$ (3) | 8.4 \pm 2.9 $\times 10^{-7}$ | 67 | 1.1 \pm 0.6 $\times 10^{-6}$ (2) | 6.7 \pm 3.5 $\times 10^{-7}$ | 12 |
| Imidacloprid | 1.9 \pm 0.6 $\times 10^{-6}$ (3) | 1.7 \pm 0.5 $\times 10^{-6}$ | 33 | 3.5 \pm 2.3 $\times 10^{-6}$ (2) | 2.1 \pm 1.4 $\times 10^{-6}$ | 4 |
| MLA | 1.5 $\times 10^{-10}$ (1) | 1.2 $\times 10^{-10}$ | 466667 | 5.2 \pm 3.1 $\times 10^{-9}$ (3) | 3.1 \pm 1.8 $\times 10^{-9}$ | 2580 |
| (-)-Nicotine | 2.3 \pm 0.5 $\times 10^{-6}$ (4) | 2.0 \pm 0.4 $\times 10^{-6}$ | 28 | 2.6 \pm 1.4 $\times 10^{-6}$ (4) | 1.6 \pm 0.8 $\times 10^{-6}$ | 5 |

Table 3.4. Parameters for the inhibition of specific [125 I] α -btx binding by cholinergic ligands. Values derived as described in section 3.3.5, for the mean \pm sem for 3 to 4 or mean \pm range for 2 independent assays (except MLA, adults), where numbers in parentheses represent the number of assays. *adults, K_i calculated assuming K_d of 6.5nM for [125 I] α -btx binding and an [125 I] α -btx concentration of 1nM, ■larvae, K_i calculated assuming K_d of 7.6nM for [125 I] α -btx binding and an [125 I] α -btx concentration of 5nM.



a)



b)

Figure 3.7. Competition binding assays of cholinergic ligands at the [125 I] α -btx binding site in a) adults and b) larvae. Increasing concentrations of ACh (\bullet), (\pm)anabasine (\blacksquare), (\pm)epibatidine (\blacklozenge), imidacloprid (\bullet), MLA (\blacksquare) and (-)-nicotine (\blacklozenge) were pre-incubated with P2 membranes for 10min before incubation with [125 I] α -btx for 1h, as described in section 3.3.3.4. Total and non-specific [125 I] α -btx binding were determined by the incubation of P2 membranes and [125 I] α -btx binding in the absence and presence of excess unlabelled α -btx respectively. Bound and free [125 I] α -btx were separated by vacuum filtration on GF/C filters. Points represent specific [125 I] α -btx binding for the mean \pm sem for 3 to 4 or mean \pm range for 2 independent assays (except MLA, adults, $n=1$). IC_{50} values obtained for each set of data were used to calculate the inhibition constant, K_i , for each drug. Mean data were fitted to the Hill equation.

For adults, the endogenous ligand, ACh, was the least potent at inhibiting [125 I] α -btx binding, with a K_i of 56 μ M. Marginally greater potency was displayed by (\pm)anabasine, with a K_i of 36 μ M. In larvae however, ACh demonstrated greater potency than (\pm)anabasine (K_i values of 8.0 μ M and 160 μ M respectively). (-)Nicotine and imidacloprid exhibited similar potency of inhibition, approximately 30-fold greater than ACh for adults and approximately 4-fold greater than ACh for larvae (Table 3.4). (\pm)Epibatidine was approximately twice as effective as nicotine and imidacloprid. Greatest potency of all was exhibited by the two cholinergic antagonists tested, α -btx and MLA. For adults, α -btx (K_i 1.3nM) was approximately 40000-fold more potent than ACh and MLA (K_i 0.12nM) was approximately 460000-fold more potent than ACh. K_i values for larvae were lower being 9.7nM for α -btx and 3nM for MLA, however, these were still 800-fold and 2500-fold more potent than ACh.

Therefore, rank orders of potency were:

for adults,

MLA > α -btx > (\pm)epibatidine > imidacloprid = (-)nicotine > (\pm)anabasine = ACh

and for larvae,

MLA > α -btx > (\pm)epibatidine > imidacloprid = (-)nicotine > ACh > (\pm)anabasine.

The greatest discrimination between adults and larvae does not appear to be with (-) nicotine, as has been hypothesised (see Chapter 1, section 1.1.3 and section 3.1.2). Accordingly the K_i values for each drug between adults and larvae were tested for statistical significance using a paired Student's t-test. At the 5% probability level, using the null hypothesis that the two K_i values from adults and larvae were not statistically different (i.e. $P > 0.05$), it was found that K_i values between adults and larvae were statistically different for the ligands ACh and (\pm)epibatidine only. P values are shown in Table 3.5. As the data for MLA in adults was from one experiment only, statistical significance between adults and larvae could not be tested.

| LIGAND | P value |
|----------------------|---------|
| α -Btx | 0.16 |
| ACh | 0.04 |
| (\pm)Anabasine | 0.30 |
| (\pm)Epibatidine | 0.03 |
| Imidacloprid | 0.89 |
| MLA | ND |
| (-)Nicotine | 0.60 |

Table 3.5. Statistical significance of K_i values for cholinergic ligands between adults and larvae. For statistical significance $P < 0.05$. ND is not determined.

The α -btx binding data generated a dose response curve that was steeper than those for the other ligands (see Figures 3.4 and 3.7) and this was reflected in the Hill numbers (nH) obtained, as shown in Table 3.6. For adults, the α -btx Hill number is close to unity as opposed to those for the other ligands which are closer to 0.4. For larvae, the α -btx Hill number is slightly less than one and for the other ligands is close to 0.2.

| LIGAND | ADULTS nH | LARVAE nH |
|----------------------|---------------------|---------------------|
| α -Btx | 0.92 \pm 0.08 (3) | 0.68 \pm 0.11 (3) |
| ACh | 0.43 \pm 0.03 (3) | 0.23 \pm 0.05 (3) |
| (\pm)Anabasine | 0.52 \pm 0.05 (3) | 0.13 \pm 0.06 (3) |
| (\pm)Epibatidine | 0.46 \pm 0.09 (3) | 0.26 \pm 0.04 (2) |
| Imidacloprid | 0.44 \pm 0.02 (3) | 0.13 \pm 0.08 (2) |
| MLA | 0.33 (1) | 0.24 \pm 0.02 (3) |
| (-)-Nicotine | 0.39 \pm 0.03 (4) | 0.28 \pm 0.03 (4) |

Table 3.6. Hill numbers (nH) derived from inhibition of specific [125 I] α -btx binding by cholinergic ligands in adults and larvae. Values are the mean \pm sem for 3 to 4 or mean \pm range for 2 independent assays (except MLA, adults) where numbers in parentheses represent the number of assays.

Inhibition by muscarinic ligands

The inhibition of [125 I] α -btx binding by muscarinic ligands is shown in Figure 3.8. At a concentration of 100 μ M, the muscarinic antagonist atropine inhibited specific binding of [125 I] α -btx more in adults than in larvae (67% compared with 61%). In contrast, the definitive muscarinic agonist muscarine showed ~27% inhibition for adults but ~66% inhibition in larvae. The same pattern was observed for oxotremorine (45% inhibition for adults and 71% inhibition for larvae).

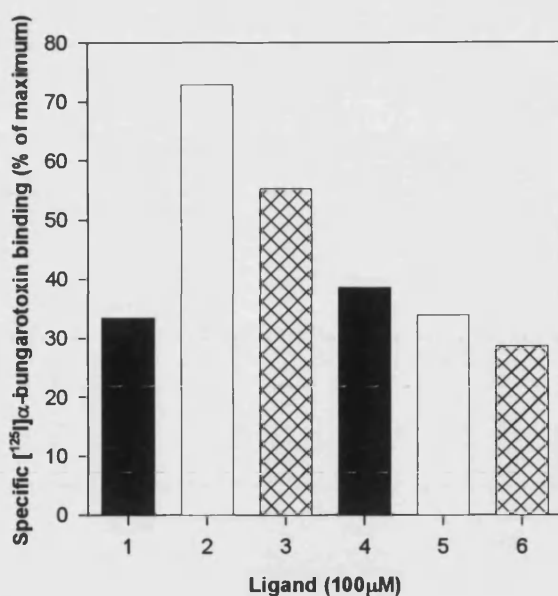


Figure 3.8. Inhibition of specific [125 I] α -btX binding by muscarinic ligands for adults (1-3) and for larvae (4-6). P2 membranes were pre-incubated with the muscarinic ligands atropine (1 and 4), muscarine (2 and 5) and oxotremorine (3 and 6) for 10min before incubation with [125 I] α -btX for 1h, as described in section 3.3.3.4. Total and non-specific [125 I] α -btX binding were determined by the absence and presence of excess unlabelled α -btX respectively. Bound and free [125 I] α -btX were separated by vacuum filtration on GF/C filters.

3.4.4 [3 H](\pm)Epibatidine competition binding

[3 H](\pm)Epibatidine binding to adult and larval membranes is shown in Figure 3.9. Almost the same profile is seen between adults and larvae. As expected, the number of counts bound increased from 1nM to 10nM [3 H](\pm)epibatidine. However, in all cases, the total binding counts and those obtained with excess unlabelled (-)nicotine or α -btX were very similar. Statistical analysis using a paired Student's t-test shows that, with the exception of 1nM [3 H](\pm)epibatidine and 1mM nicotine for larvae where $P = 0.045$, there is no statistical difference between total binding and displacement binding with either 1mM (-) nicotine or 1μM or 5μM α -btX (for adults and larvae respectively) ($P > 0.05$). Controls without P2 membranes showed slightly lower counts for 1nM [3 H](\pm)epibatidine but varied for 10nM epibatidine.

Previously, comparison of the total counts of [125 I] α -btX in a sample, with the total [125 I] α -btX counts bound to P2 membranes, had indicated that a maximum of 4% of the total [125 I] α -btX counts bound to the membranes. If the same applied to [3 H](\pm)epibatidine, total counts bound for 1nM [3 H](\pm)epibatidine using adults should have been ~370cpm, as opposed to the 25cpm recorded in the experiment. Furthermore, statistical analysis using a paired Student's t-test indicated that a difference of 13cpm between total and non-specific binding (i.e. specific binding) was necessary for statistical significance ($P < 0.05$). This was equivalent to 0.28fmol of binding sites. Assuming that 1mM nicotine was in excess and acted as a non-specific ligand, specific binding was 0.36fmol/mg.

This suggests that the number of binding sites were not below the limit of detection of the assay. The combination of these factors suggest therefore, that there is not a [^3H](\pm)epibatidine binding site in *Manduca* membranes, and that the binding detected is non-specific binding.

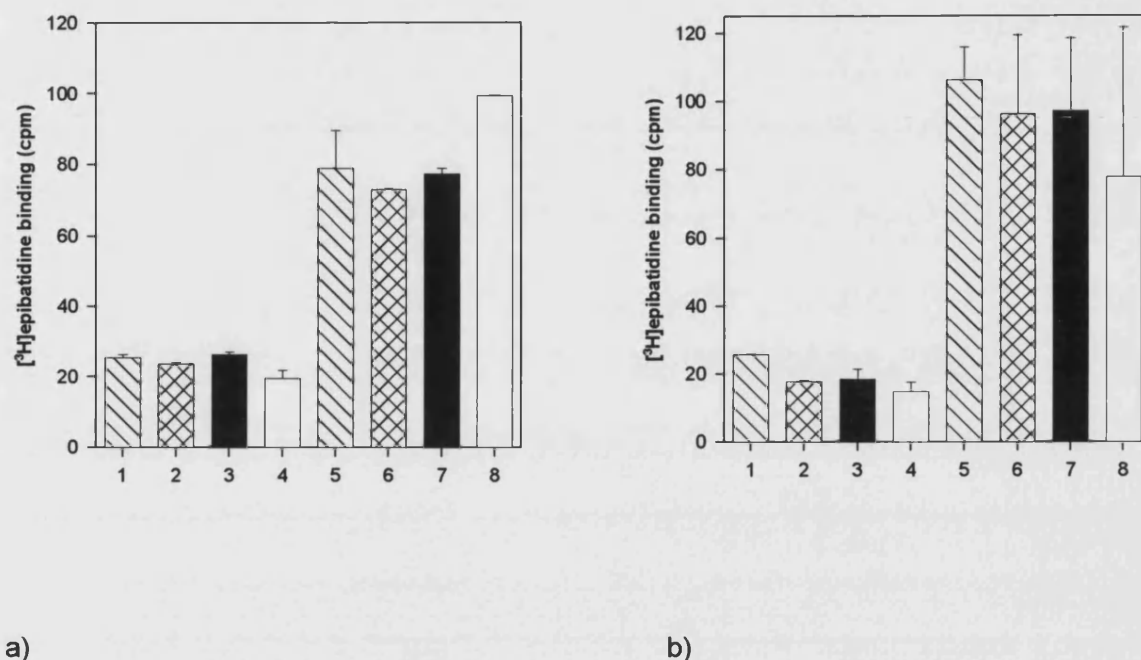


Figure 3.9. Inhibition of [^3H](\pm)epibatidine binding by excess (-)nicotine and α -btx in a) adult and b) larval membranes. P2 membranes were pre-incubated with excess unlabelled (-) nicotine or α -btx for 10min before incubation with [^3H](\pm)epibatidine for 1h, as described in section 3.3.4. Total [^3H](\pm)epibatidine bound was determined by incubation of P2 membranes and [^3H](\pm)epibatidine for 1h. Bound and free [^3H](\pm)epibatidine were separated by vacuum filtration on GF/C filters. Total bound: 1nM [^3H](\pm)epibatidine (1); non-specific bound: 1nM [^3H](\pm)epibatidine with 1mM nicotine (2); non-specific bound: 1nM [^3H](\pm)epibatidine with 1 μM (in adults) or 5 μM (in larvae) α -btx (3); control: 1nM [^3H](\pm)epibatidine without membrane preparation (4); Total bound: 10nM [^3H](\pm)epibatidine (5); non-specific bound: 10nM [^3H](\pm)epibatidine with 1mM nicotine (6); non-specific bound: 10nM [^3H](\pm)epibatidine with 1 μM (in adults) or 5 μM (in larvae) α -btx (7) and control: 10nM [^3H](\pm)epibatidine without membrane preparation. Bars are mean \pm range of 2 assays.

3.5 Discussion

[¹²⁵I]α-Btx displayed saturable and high affinity binding to *Manduca* tissue consistent with the presence of α-btx sensitive AChRs and thus agreed with previous evidence from *Manduca* presented by Sanes *et al.* (1977) and Hildebrand *et al.* (1979).

Saturability of α-Btx binding sites

The number of α-btx binding sites increased ~7-fold from larvae to adults, as seen in Table 3.7. Comparison with other insects (Table 3.7) shows that *Manduca* larvae have fewer binding sites than *Apis* (Tomizawa *et al.*, 1995a) while both larvae and adults have fewer binding sites than *Schistocerca* (MacAllan *et al.*, 1988). As association and dissociation rates were not measured, the apparent K_d s were estimated from the concentrations of free toxin required for half saturation. K_d values for *Manduca* were close to those of *Apis* (Tomizawa *et al.*, 1995a) but higher than that from *Schistocerca* (MacAllan *et al.*, 1988). Scatchard analysis revealed one binding site, unlike the most recent Scatchard analysis on *Drosophila* which revealed two binding sites with B_{max} values of 240fmol/mg protein and 1080fmol/mg protein and K_d values of ~0.1nM and ~4nM respectively (Schloss *et al.*, 1988).

| | <i>Manduca</i> adults | <i>Manduca</i> larvae | <i>Apis</i> | <i>Schistocerca</i> |
|--------------------------------|--------------------------|--------------------------|-------------|---------------------|
| B_{max} (fmol/mg protein) | 815 | 119 | 451 | 1200 |
| K_d (nM) | 6.5 | 7.6 | 7.5 | 0.8 |

Table 3.7. Comparison of B_{max} and K_d for *Manduca*, *Apis* (Tomizawa *et al.*, 1995a) and *Schistocerca* (MacAllan *et al.*, 1988).

Metabolism of nicotine by the central nervous system in Manduca

Morris (1983a) established that the CNS of *Manduca* contained a local detoxification system, thus enabling it to tolerate nicotine (see Chapter 1, section 1.1.3). Therefore, it can be hypothesised that the presence of metabolic enzymes such as cytochrome P450 results in the breakdown of nicotine before interaction with nAChRs results in desensitisation and death. To assess the possibility of nicotine breakdown and its subsequent effects on the inhibition of specific [¹²⁵I]α-btx binding, three experiments were performed using various pre-incubation times. If metabolism by CNS detoxifying enzymes played a major role in nicotine insensitivity, a clearly defined pattern in which

the longer the total incubation time of nicotine with membrane preparation, the greater the breakdown of nicotine and the lower the affinity for binding to the α -btx binding site would be expected. However, the results obtained (Figure 3.5) suggest that the pre-incubation of nicotine has little effect on the inhibition of [125 I] α -btx binding over the 1h incubation period used. Admittedly, the binding affinity of nicotine for the α -btx site decreases slightly with an increase in pre-incubation time (Table 3.2). There is approximately a 3-fold difference between no pre-incubation at 4°C (plus 60min incubation) and 60min pre-incubation at room temperature (plus 60min incubation), though the difference with 10min pre-incubation at room temperature (plus 60min incubation) of approximately 1.5-fold is much less marked. This suggests that there is some possibility of nicotine metabolism by the membrane preparation but at the 10min pre-incubation period (plus 60min incubation) used subsequently, there is not sufficient breakdown to make nicotine binding any different from other cholinergic ligand binding. It is therefore significant that Morris (1983a) observed more than half the nicotine taken up at the concentration of 10^{-4} M was metabolised within 5min, which clearly is not the case if measurement is taken as the inhibition of [125 I] α -btx binding.

If nicotine metabolism by the CNS of *Manduca* did play a major role in preventing the binding of nicotine to nAChRs in *Manduca*, hence reducing *Manduca*'s nicotine sensitivity, the dose response curves shown would be expected to show a distinct shift to the right and this has not happened. It is always possible that the enzymes responsible for the nicotine metabolism were destroyed or washed out during the preparation of the membranes for some inexplicable reason or that rapid metabolism of nicotine occurred in which the resulting breakdown product displaced the specific binding of radiolabelled α -btx with similar affinities at each pre-incubation time.

Although the structures of nicotine and nicotine-1-N-oxide (the nicotine metabolite found in the CNS; Snyder *et al.*, 1993; see Chapter 1, section 1.1.3) are similar, the process of metabolising a potentially toxic compound (nicotine) into another compound that had high affinity for the same target and hence also toxic, seems extremely maladaptive. However, if nicotine is toxic and the metabolic product is not, similar nicotine binding affinities to other nicotine sensitive insects would not be expected. It is of relevance that the CNS of *Periplaneta*, a nicotine sensitive insect was also shown to metabolise nicotine, although to a lesser extent than *Manduca* (Morris, 1983a) indicating that this metabolic process was not unique to *Manduca*.

These preliminary results also suggested that the binding of nicotine to the α -btx sensitive nAChR in *Manduca* was not especially different from that in nicotine sensitive insects (see Comparison with other insects below), implying that other mechanisms are essential for the insensitivity to nicotine seen in *Manduca* (discussed in Chapter 4).

Pharmacological specificity

Inhibition of [125 I] α -btx binding in *Manduca* resulted in a similar pharmacological profile for both adults and larvae with the antagonists tested, binding with nanomolar affinities and the agonists tested, binding with micromolar affinities.

Statistical analysis between adults and larvae indicated that there were differences in binding affinities for the ligands ACh and (\pm)epibatidine only (Table 3.5). For (\pm)epibatidine binding, the statistical difference obtained may be due to the lower number of binding assays for larvae compared with adults, as this error is taken into account in the calculation. The difference between adults and larvae for ACh is more difficult, if not impossible to explain. Adults exhibit lower binding affinity for the site and why this is so remains unclear. Despite the inclusion of the AChE inhibitor, neostigmine in all ACh assays, it is possible that ACh was partially metabolised in the adult preparations and therefore resulted in lower binding affinity.

Previously, it has been hypothesised that a different nAChR may exist for adults and larvae (see Chapter 1, section 1.1.3 and section 3.1.2). To recap, as larvae feed on tobacco without suffering any detrimental effects from nicotine poisoning, they may possess a nAChR that is altered within its agonist binding site (in comparison with other insects and / or vertebrates) such that nicotine fails to bind. Adults on the other hand, feed on nectar. Thus, the adult diet is devoid of nicotine and consequently a nAChR that does not bind nicotine is not necessary. If this were the case, it could be argued that there should be a statistical difference between adults and larvae for the binding affinity of nicotine. As the larvae nAChR would be nicotine insensitive, a low binding affinity would be observed whereas a nicotine sensitive nAChR in adults would result in a higher binding affinity. Predictably, a large evolutionary selection pressure would be needed to change a nicotine insensitive receptor to a nicotine sensitive receptor as this would be maladaptive. It is not surprising therefore, that the nicotine binding affinities for adults and larvae are almost identical (2.0 μ M and 1.6 μ M respectively). These results suggest that the same α -btx binding site is maintained throughout the lifecycle of *Manduca*.

Nevertheless, it could be argued that as the larvae were fed on an artificial diet, they did not have any nicotine resistance and so have the same apparent binding affinity as the adults. However, *Manduca* larvae from the University of Bath *Manduca* colony have previously demonstrated nicotine resistance from their ability to feed and grow normally on tobacco plants (Reynolds *et al.*, 1986). Moreover, Trimmer and Weeks (1989) have proven that resistance to nicotine is an intrinsic rather than an inducible trait, as larvae in their *Manduca* colony do not show increased resistance when raised on tobacco plants or nicotine-laced diets.

Comparison with other insects

Although it appears that adults and larvae share the same α -btx binding site, it is necessary to compare the binding affinities obtained with those from other insects to deduce the presence of nicotine or other cholinergic ligand resistance. Table 3.8 shows K_i values for insects covered in the literature. As these literature values use adult tissues, *Manduca* adult data is shown for comparison in the table. In fact studies on larval forms are rare (Osborne, 1996).

The nicotinoids - nicotine and anabasine

It is pertinent here to address the question of nicotine insensitivity among other insects. With the exception of *Manduca*, the insects shown in Table 3.8 are not considered to be nicotine insensitive. Studies on *Apis* and *Periplaneta*, the two insects with the closest nicotine binding affinity to *Manduca*, show that nicotine is acutely toxic. In *Apis*, nicotine poisoning begins with stupefaction and staggering followed by paralysis of legs, wings, antennae and mandibles, suggesting that nicotine causes ascending motor paralysis of the nerve cord (reviewed in M.E. Eldefrawi, 1985). In *Periplaneta*, high concentrations of nicotine appear to cause complete depression and paralysis of the heart (reviewed in M.E. Eldefrawi, 1985). In contrast, injection of nicotine into the haemolymph of *Manduca* does not cause toxic effects - indeed *Manduca* resumes normal feeding habits after 30min (Self *et al.*, 1964a). Comparison with other lepidopterans shows that this is not a lepidopteran specific effect as *Pieris brassicae* is killed by nicotine in its diet (David and Gardiner, 1953). As reviewed in Chapter 1 (section 1.1.1), these toxic effects are primarily caused by the interaction of nicotine with nAChRs. Therefore, it is clear that the nicotine binding affinity seen in *Manduca* is comparable with those from other insects and does not suggest that the α -btx binding site imparts the nicotine insensitivity seen in *Manduca*.

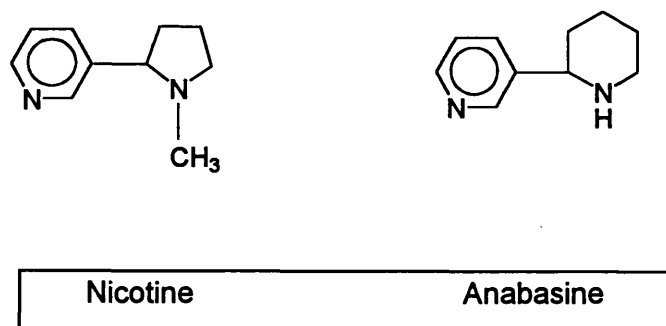
| LIGAND | <i>Manduca</i> adults | <i>Drosophila</i> | <i>Musca</i> | <i>Apis</i> | <i>Periplaneta</i> | <i>Schistocerca</i> | <i>Locusta</i> |
|---------------|--------------------------|----------------------|-------------------------|----------------------|---|---------------------------|-----------------------|
| α -Btx | 1.3×10^{-9} | 1.5×10^{-9} | 10^{-9} | 5.5×10^{-9} | 8.5×10^{-10} | 8.9×10^{-9} | 1.8×10^{-10} |
| ACh | 5.6×10^{-5} | 1.2×10^{-6} | 1.8×10^{-5} | ND | 1.8×10^{-5} | **** 2.7×10^{-6} | 1.1×10^{-4} |
| Anabasine | 3.6×10^{-5} | ND | ND | 1.6×10^{-6} | ND | ND | ND |
| Epibatidine | 8.4×10^{-7} | ND | ND | ND | ND | ND | ND |
| Imidacloprid | 1.7×10^{-6} | ND | ND | 1.5×10^{-6} | ** 2×10^{-7} | ND | ND |
| MLA | 1.2×10^{-10} | ND | * 2.5×10^{-10} | ND | *** 1.4×10^{-9} | 1.8×10^{-8} | ND |
| (-)-Nicotine | 2.0×10^{-6} | 1.4×10^{-7} | 9.0×10^{-7} | 1.2×10^{-6} | 3.5×10^{-6} ** 9.8×10^{-6} | 5.1×10^{-7} | 1.1×10^{-8} |

Table 3.8. Comparison of *Manduca* K_i values with those from *Drosophila* (Schmidt-Nielsen *et al.*, 1977: K_i values calculated from IC_{50} values quoted, using K_d value of 1.1nM and various [125 I] α -btx] also quoted), ***Musca*** (Cattell *et al.*, 1980a: IC_{50} values quoted; *Jennings *et al.*, 1986), ***Apis*** (Tomizawa *et al.*, 1995a: K_i values calculated from IC_{50} values quoted, using K_d value of 7.5nM and [125 I] α -btx] of 2nM also quoted), ***Periplaneta*** (Lummis and Sattelle, 1985; **Bai *et al.*, 1991: IC_{50} values quoted; ***Sattelle *et al.*, 1989), ***Schistocerca*** (MacAllan *et al.*, 1988; ****Filbin *et al.*, 1983: K_i value calculated from IC_{50} value quoted, using K_d value of 1.38nM and [125 I] α -btx] of 2nM also quoted), and ***Locusta*** (Breer, 1981: K_i values calculated from IC_{50} values quoted, using K_d value of 1.1nM and [125 I] α -btx] of 3.1nM also quoted).

Naturally occurring nicotine analogues, termed nicotinoids, include anabasine - another alkaloid found in tobacco. It is conceivable therefore, that the nicotine insensitivity seen in *Manduca* is just part of a spectrum encompassing insensitivity to many alkaloids. As *Manduca* can feed on a wide range of solanaceous plants, it is more likely that a general mechanism exists that prevents alkaloid toxicity.

It has been established that the nicotine insensitivity seen in *Manduca* is probably not due to a mutated nAChR containing an α -btx binding site, as similar binding affinities are seen with nicotine sensitive insects. Comparison with anabasine binding affinities however, are not as convincing. Although comparison is limited to one insect, *Apis* (Tomizawa *et al.*, 1995a), it appears that anabasine has ~20-fold higher binding affinity for the α -btx binding site in *Apis* than *Manduca* adults (increasing further with comparison of *Manduca* larvae). Taken as a single result, out of context, this could suggest that anabasine binding is reduced due to a receptor that does not form tight bonds with anabasine. Considering that nicotine, the primary alkaloidal target of this investigation does bind with a similar extent as other insects, another explanation is warranted.

Nicotine and anabasine are exceedingly similar in structure, as shown below and thus might be expected to exhibit similar binding affinities. However, in *Manduca* adults, anabasine is ~20-fold less potent than nicotine and in *Manduca* larvae is ~100-fold less potent than nicotine. This is surprising as Campbell *et al.* (1933) demonstrated that nicotine was only 2.6-fold more toxic than anabasine on larvae from the mosquito *Culex pipiens* and in *Apis* is only 1.3-fold more potent than anabasine. Vertebrate comparison using rat gives K_i values of $0.82\mu\text{M}$ and $0.34\mu\text{M}$ for nicotine and anabasine respectively at the $[^{125}\text{I}]\alpha\text{-btX}$ binding site (Marks *et al.*, 1986) demonstrating in this instance that anabasine is slightly more potent than nicotine. This suggests that there is an element of experimental error within the *Manduca* binding data. It has already been noted that the standard errors of the larvae assays are large due to the lower purity of the membrane preparation and for the anabasine assay only, this results in a 3-fold disparity between IC_{50} values obtained from the individual experiments and the curve fit data. Furthermore, the anabasine obtained from Sigma was the last of the Company's existing stocks and no alternative sources were available. This supply appeared to have been synthesised some years earlier and quite possibly had gone off. The solution was yellow and previous experience with nicotine had shown that a discoloured nicotine solution resulted in lower binding affinity than usual. These observations may account for the differences seen between anabasine and nicotine in *Manduca*. However, it remains possible that in *Manduca* anabasine is less potent than nicotine, suggesting that different residues are involved in the binding of nicotine and anabasine.



Although detailed structure-activity relationships for nicotinic agonists on the nAChR have been carried out on vertebrates (Holladay *et al.*, 1995), corresponding relationships in insects are scarce. Studies relating insecticidal activity to structural features (reviewed in Yamamoto, 1966) have shown that both nicotine and anabasine are highly toxic and that this is due to several structural features of the nicotinoids. The pyridine ring plays an important role in toxicity but alone is not sufficient, in addition an amino nitrogen in the side chain group must be separated from the pyridine ring by a definite distance. This is

achieved by the attachment of the 3-position of the pyridine ring to the 2-position of the pyrrolidine ring in nicotine or the 2-position of the piperidine ring in anabasine, resulting in a minimum distance between the pyridyl nitrogen and the pyrrolidyl or piperidyl nitrogen of the two rings of 4.2Å. It appears that the stereochemical arrangement of the pyridine and the pyrrolidyl or piperidyl nitrogen moieties is necessary for exerting the action on the receptor. Furthermore, it is necessary for the amino nitrogen to be of high basicity (pK_{a1} , 7.4-9.0) as this results in the monocationic form of the nicotinoid (ionised at the pyrrolidine or piperidine nitrogen) acting at the receptor binding site and appears to have higher toxicity than the free base.

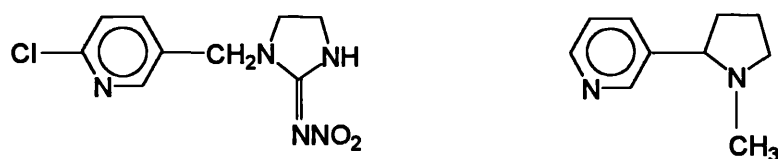
These features are in general agreement with the requirements of a nicotinic pharmacophore as described by Beers and Reich (1970). In this model, it is proposed that nicotine requires the alkylammonium moiety for an electrostatic charge-charge interaction with the nAChR and an hydrogen bond acceptor group (the nitrogen on the pyridine ring) which is ~5.9Å from the centre of positive charge on the alkylammonium moiety (compared with the minimum distance of 4.2Å calculated by Yamamoto). Furthermore, it is proposed that the carbon skeleton provides the correct stereochemical localisation of the functional groups determining specificity.

Neonicotinoids - imidacloprid

Recent binding studies have revealed that nicotine and the nitroguanidine analogue, 1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine (imidacloprid) have differing selective toxicity in vertebrates and insects (Yamamoto *et al.*, 1995). As imidacloprid is a cholinergic acting insecticide (Abbink, 1991) it was used in *Manduca* binding studies. Data presented in this chapter (Table 3.8) show that the binding affinity of imidacloprid is similar for *Manduca* adults (and larvae) and *Apis* (Tomizawa *et al.*, 1995a). Furthermore, the binding affinities of imidacloprid and nicotine are almost equal in both species. In contrast, inhibition of [125 I] α -btx binding in *Periplaneta* showed approximately a 50-fold increase in binding affinity of imidacloprid compared with nicotine (Bai *et al.*, 1991). Imidacloprid binding at the vertebrate site in *Torpedo* (equivalent to the muscle type nAChR) however, shows a marked lack of affinity for the α -btx binding site (Tomizawa *et al.*, 1995b) demonstrating ~15-fold lower binding affinity than nicotine, and ~500-fold lower binding affinity than imidacloprid in *Apis* (Tomizawa *et al.*, 1995a).

Yamamoto *et al.* (1995) have therefore suggested that imidacloprid is one of the few cholinergic ligands that can distinguish between vertebrate and invertebrate targets and hence is ideal as an insecticide. They have postulated the following mechanism of action. Imidacloprid, shown below, possesses an electron-withdrawing nitroimine group

and this delocalises the unshared electron pair on the nitrogen atom corresponding to the amino nitrogen atom of nicotine and becomes partially positive. This partially positive nitrogen atom is sufficient to interact strongly with the insect nAChR but not with the vertebrate nAChR which requires a fully positive charge.



Imidacloprid

Nicotine

Nicotine is not an especially useful insecticide, as at physiological pH (generally above pH6.5) it is highly ionised and therefore cannot penetrate either the insect body or through an ion or blood-brain barrier into the synapse. Imidacloprid, on the other hand, is not highly ionised and thus can penetrate to the CNS and interact with the nAChR causing insecticidal activity. In vertebrates, it is not easy for ionised nicotine to cross the blood-brain barrier and interact with the receptors of the CNS (in contrast to lipophilic nicotine). However, nicotine can interact with the peripheral nAChRs whereas, imidacloprid, due to its lack of total positive charge is less capable of interaction with receptors either in the PNS or when it has crossed the blood-brain barrier to the receptors in the CNS.

Given that *Manduca* appear to survive the ingestion of vast quantities of nicotinoids, it would be highly applicable to test for the 'Manducacidal' activity of imidacloprid. The evidence so far obtained does not suggest a cholinergic receptor with properties different from other susceptible insects. It would therefore be important to show that a cholinergic compound such as imidacloprid could bind to the nAChRs in *Manduca* without being eliminated in the same manner as nicotine.

Interestingly, imidacloprid possesses a chloropyridine group similar to epibatidine, which in *Manduca* is twice as potent as imidacloprid. In structure-activity studies using epibatidine analogues, replacement of the chloro moiety with hydrogen did not affect the binding affinity for [³H]nicotine sites in rat brain membranes whereas replacement with a methyl or iodo moiety resulted in lower binding affinity (Badio and Daly, 1994). It is possible that the same substitutions could affect binding affinity in imidacloprid. Epibatidine binding in *Manduca* is discussed in detail later.

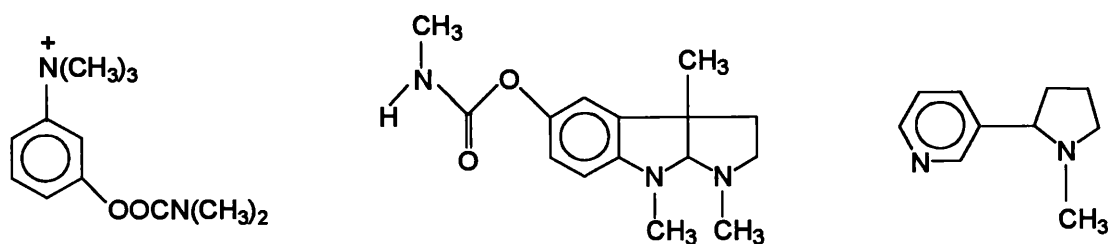
Acetylcholine and acetylcholinesterase inhibitors

It was important to test the binding affinity of ACh to make sure that the α -btx binding site was cholinergic in nature. If nicotine had not bound as well as these experiments suggest, ACh would have been used as a reference point. It can be seen that in adult tissues, the binding affinity of ACh is consistently an order of magnitude lower than (-) nicotine (except for *Locusta migratoria* where excessive discrimination is observed; Breer, 1981). It appears therefore, that as a quaternary amine, ACh is not as effective as nicotine - a tertiary amine.

It was necessary to supplement ACh assays with AChE inhibitors, thus preventing breakdown of ACh from the AChE present in the membrane preparations. A preliminary experiment evaluated several AChE inhibitors for their lack of ability in binding to the α -btx binding site, at a concentration (10 μ M) high enough to stop degradation of ACh. Neostigmine was the least effective in inhibiting binding of [¹²⁵I] α -btx whilst BW284c51, THA and physostigmine had an increasing tendency to bind to the α -btx binding site.

AChE inhibitors have been used as insecticides for many years and thus the finding that they can also bind to α -btx binding sites in insects may make some contribution to their insecticidal activity. ACh interacts with AChE at two sites - an anionic site which attracts the quaternary N atom by electrostatic force and an esteratic site, comprising a basic nucleophilic imidazole group of histidine and an hydroxyl group of serine, the latter attracting the electrophilic carbonyl C atom in a two-stage reaction. Firstly, an enzyme-substrate complex is formed before acetylation of the enzyme and release of the choline. Secondly, the electronegative O atom of a water molecule attacks the electrophilic C atom of the acetyl group, regenerating the active enzyme and producing acetic acid.

Neostigmine and physostigmine, as shown below, are AChE inhibitors of the carbamate type, binding to the esteratic subsite of the AChE in a slowly reversible manner. They carbamylate the serine hydroxyl that ACh acetylates (A.T. Eldefrawi, 1985). Neostigmine possesses a quaternary ammonium group which also interacts with the anionic site of AChE and is considered to be more stable than physostigmine.



Neostigmine

Physostigmine

Nicotine

Physostigmine, has been shown to have both an agonistic and channel blocking effect on the nAChR in vertebrates (Shaw *et al.*, 1985). Studies on vertebrates have shown that physostigmine acts as a non-competitive agonist (Storch *et al.*, 1995), binding to sites that are distinct from those for ACh (Schröder *et al.*, 1994). The carbamate group is not required for agonistic action on nAChRs (Okonjo *et al.*, 1991). In the α subunit of the nAChR, a conserved lysine residue (K125 in *Torpedo*; see Appendix 1), three before the first cys of the cys loop is photoaffinity labelled by [3H]physostigmine (Schrattenholz *et al.*, 1993) but has not been shown to be involved in ACh binding (see Chapter 1, section 1.2.7). Schröder *et al.* (1994) report that the region α 118-137 in *Torpedo* contains the physostigmine binding site. This overlaps with one of several regions thought to bind α -btx, namely *Torpedo* α 122-150 (Mulac-Jericevic and Atassi, 1987a,b; see Chapter 1, section 1.2.7), and may explain why physostigmine binds to the α -btx binding site, although it has been shown that activation of the nAChR by physostigmine is insensitive to α -btx (Okonjo *et al.*, 1991).

Comparison of physostigmine with nicotine, as shown above, indicates that the presence of an N-methyl group attached to a pyrrolidine ring in both structures may serve an important function in binding to the α -btx binding site. In comparison, neostigmine, which binds with much lower binding affinity than either nicotine or physostigmine does not possess this group. In fact, Schmidt-Nielsen *et al.* (1977) showed that neostigmine was approximately 1000-fold less potent than physostigmine, which had nearly identical binding affinity with ACh at the α -btx binding site in solubilised *Drosophila* membranes. Similarly, in *Manduca* membranes at the 10 μ M concentration used, neostigmine had a negligible effect on the inhibition of [^{125}I] α -btx binding whereas physostigmine was very effective.

Thus, the capacity for neostigmine as an insecticide may lie exclusively in its ability to inhibit AChE. However, if the agonistic action of physostigmine in vertebrates is cautiously extrapolated to insects, the similar potential for insecticidal activity by physostigmine may be offset by its enhancing activity as a non-competitive agonist. Nevertheless, physostigmine is known to be at least as potent an insecticide as neostigmine and this may be due to its ability to block the nAChR ion channel at micromolar concentrations as shown in vertebrates (Shaw *et al.*, 1985).

THA is an important inhibitor of AChE, being the only anticholinesterase licensed for the treatment of Alzheimer's Disease (Kellar and Wonnacott, 1990). Perry *et al.* (1988) suggested that the success of THA was related to its interactions with the nAChR (and possibly the mAChR) rather than its properties as an AChE inhibitor. It was shown that THA was 1000-fold more potent than physostigmine in inhibiting [³H]nicotine binding in human brain. THA clearly does not have the same potency in *Manduca* where it is less potent than physostigmine (Table 3.3). Moreover, in *Manduca*, the AChE inhibitor BW284c51 had lower potency than THA but was more potent than neostigmine. This difference in potency may be related to their differences in structure. THA and Bw284c51 are unlike each other and also differ from neostigmine and physostigmine. Indeed, THA does not even possess a carbamate moiety.

Binding of antagonists

The two antagonists studied were by far the most potent ligands in inhibiting the binding of radiolabelled α -btx, with MLA being more potent than α -btx in both *Manduca* adults and larvae. As all agonists are small in size, it is presumed that competitive antagonists overlap the agonist site of action. Indeed, there are a number of regions that appear to be important in α -btx binding, those that are discriminatory being found in the muscle type nAChR and the neuronal $\alpha 7$ and $\alpha 8$ (and maybe $\alpha 9$) subunits (see Chapter 1, section 1.2.7).

MLA is found in seeds of *Delphinium brownii* (Nambi-Aiyar *et al.*, 1979) and has come to prominence as an $\alpha 7$ selective ligand. In vertebrates, it shows ~1000-fold selectivity for the neuronal [¹²⁵I] α -btx binding site compared with neuronal [³H]nicotine binding sites and neuromuscular [¹²⁵I] α -btx binding sites (MacAllan *et al.*, 1988; Ward *et al.*, 1990). Furthermore, expression in *Xenopus* oocytes has shown that MLA blocks $\alpha 3\beta 2$ and $\alpha 4\beta 2$ receptors with low micromolar affinity but $\alpha 7$ receptors with picomolar affinity, reinforcing the hypothesis that the [¹²⁵I] α -btx binding site is a property of the $\alpha 7$ subunit (Wonnacott *et al.*, 1993; Palma *et al.*, 1996).

From Table 3.8, it can be seen that MLA and α -btx show very similar high affinity binding for α -btx binding sites in insects. The MLA binding affinity for *Manduca* ($K_i = 0.12\text{nM}$) is very comparable with the most evolutionarily related species shown, *Musca* ($K_i = 0.25\text{nM}$; Jennings *et al.*, 1986) although MLA is less potent in *Periplaneta* (Sattelle *et al.*, 1989) and *Schistocerca* (MacAllan *et al.*, 1988). These results imply that the α -btx binding site in insects must have similar properties to that defining the vertebrate $\alpha 7$ subunit.

Structure-activity relationships using rat brain have suggested that the ester-linked 2-(methylsuccinimido)benzoyl group is necessary for nicotinic potency (corresponding to the basic structure of nicotinoids) but that the [^{125}I] α -btx binding and $\alpha 7$ selectivity reside in the norditerpenoid core (Hardick *et al.*, 1995).

Is there a [^3H](\pm)epibatidine binding site in Manduca ?

Having established that an α -btx binding site consistent with an α -btx sensitive receptor (but not proving it) was present in *Manduca* adults and larvae, the presence or absence of putative α -btx insensitive receptors was determined. Epibatidine (exo-2-(6-chloro-3-pyridyl)-7-azabicyclo-[2.2.1]heptane), from the skin of the Ecuadorian poison frog, *Epipedobates tricolour*, is the most potent nicotinic agonist discovered to date. Several studies have shown that epibatidine has extremely high picomolar affinity for the $\alpha 4\beta 2$ receptor as determined by displacement of the radioligands [^3H]nicotine (Badio and Daly, 1994) and [^3H]cytisine (Sullivan *et al.*, 1994). Contrastingly, epibatidine displays ~5000-fold lower binding affinity for the $\alpha 7$ receptor, as determined by displacement of [^{125}I] α -btx (Sullivan *et al.*, 1994). It appears therefore, that epibatidine shows selectivity toward the $\alpha 4\beta 2$ subtype of nAChR, in contrast to MLA. However, some nAChR subtypes present in the brain and ganglia may have affinities for [^3H]nicotine and [^3H]cytisine too low to be reliably labelled by these radioligands in a binding assay. Hence, the possibility of other receptor subtypes that bind epibatidine with high affinity arises (Kellar, 1995). Fortunately, [^3H](\pm)epibatidine is now commercially available and as expected has high affinity for nAChRs. Houghtling *et al.* (1995) have demonstrated that [^3H](\pm)epibatidine appears to bind to two classes of nAChR sites in rat and human brain, both with picomolar affinity. One of these is the $\alpha 4\beta 2$ receptor while the other is thought to be a site containing the $\alpha 3$ subunit.

It is possible that *Manduca* nervous tissue contained nAChR subtypes other than those labelled by α -btx. Previously, MacAllan *et al.* (1988) had demonstrated that [^3H]nicotine bound with low affinity to *Schistocerca* brain preparations and did not fully saturate over the concentration range used. Moreover, it was displaced by cholinergic ligands other

than (-)nicotine with extremely poor affinity suggesting that the binding was non-specific and that the [^3H]nicotine binding site was not a physiological nAChR. Consequently, [^3H](\pm)epibatidine was the radioligand of choice in the search for other *Manduca* neuronal subunits such as $\alpha 3$ - and $\alpha 4$ -like receptors.

As seen from Figure 3.7, (\pm)epibatidine inhibited [^{125}I] α -btx binding with slightly higher affinity than (-) nicotine, indicating that it did have pharmacological properties consistent with a cholinergic ligand. However, [^3H](\pm)epibatidine binding in *Manduca* was low and probably non-specific (Figure 3.9), as determined by statistical significance using the Student's t-test. It appears therefore, that *Manduca* does not have a [^3H](\pm)epibatidine binding site and consequently neither the equivalent of an $\alpha 4\beta 2$ nAChR nor an $\alpha 3$ containing nAChR. As yet, no data for [^3H](\pm)epibatidine binding to insects have been published.

Interpretation of Hill numbers

It is noticeable that with the exception of unlabelled α -btx, all the dose response curves obtained for cholinergic ligand displacement of [^{125}I] α -btx binding were shallow and this resulted in low Hill numbers (see Table 3.6). The shallow curves obtained may be part of a wide ranging insect phenomenon, as similarly shaped curves are obtained for some other insects (see Sattelle *et al.*, 1989; Tomizawa *et al.*, 1995a). These papers do not quote Hill numbers for the inhibition of [^{125}I] α -btx binding. The Hill number for binding of α -btx in *Manduca* adults was close to unity suggesting that α -btx bound to one site with high affinity or more than one site but with equal affinity. In addition, the Scatchard plot obtained from saturation binding of [^{125}I] α -btx was linear (see Figure 3.3), implying that the existence of two different classes of α -btx binding sites was unlikely. The same appears to hold for α -btx in larvae. Conversely, the other cholinergic ligands tested have Hill numbers at or below 0.5. This could be as a result of any of the following suggestions:

- 1) The cholinergic ligand tested could be distinguishing between two or more [^{125}I] α -btx binding sites. These could represent either a second nAChR or a second site on the same receptor. It has previously been mentioned that the [^{125}I] α -btx binding site correlates to the $\alpha 7$ subtype of nAChR in vertebrates. There is no conclusive evidence of the existence of an homomeric $\alpha 7$ receptor *in vivo* although there is little doubt that it functions successfully *in vitro* (see Chapter 1, section 1.2.5). A cautious extrapolation from vertebrates to *Manduca* suggests that an $\alpha 7$ -like nAChR may well be present, either as an homomer, an heteromer, or both in *Manduca*. An homomeric $\alpha 7$ -like receptor

would contain identical α -like subunits, suggesting that each would have the same affinity for cholinergic ligands and thus a second site on the same receptor in this case, seems unlikely. Alternatively, an heteromeric $\alpha 7$ -like receptor, if assumed to have the postulated structure of other vertebrate neuronal receptors *in vitro*, could have either two identical α -like and three identical β -like subunits or non-identical α -like and non-identical β -like subunits such as the $\alpha 4\alpha 5\beta 2$ receptor (Ramirez-Latorre *et al.*, 1996) or even the muscle receptor. In the case of an heteromeric receptor with identical α -like and identical β -like subunits, the two α -like subunits would be expected to have identical binding affinity as they would have identical flanking subunits due to the 2:3 stoichiometry. However, in the case of an heteromeric receptor with non-identical α -like and non-identical β -like subunits, differing α -like or β -like subunits would be expected to result in different binding affinities as is the case with the muscle receptor which possesses an high affinity site at the α - γ interface and a low affinity site at the α - δ interface (Pederson and Cohen, 1990; see Chapter 1, sections 1.2.5 and 1.2.7). A further possibility is that of cholinergic ligands binding to sites on two different α -btx binding receptors. If this were so, it would be expected that α -btx would bind with two differing affinities as postulated for *Schistocerca* (Filbin *et al.*, 1983) and in one *Drosophila* study (Schloss *et al.*, 1988). In *Manduca* this does not appear to happen and thus it is unlikely that there are two different α -btx binding receptors. Furthermore, all the cholinergic ligands tested fitted a one site model.

2) Negative co-operativity, where the binding of one molecule to a subunit makes it more difficult for subsequent molecules to bind to other subunits, is known to give Hill numbers lower than one. This would explain the binding of the cholinergic ligands giving shallow curves but does not account for the difference obtained with α -btx, which due to its large size would be more likely to cause negative co-operativity through steric hindrance, than the other ligands. α -Btx, as a polypeptide is by far the largest ligand tested and its binding site extends beyond that of the smaller ligands (see Chapter 1, section 1.2.7).

3) Ligand heterogeneity is a possibility for some of the ligands used. For example, the epibatidine used was racemic, presumably having equal amounts of (+) and (-). It has been shown that the naturally occurring (+) enantiomer is ~1.3-fold more potent than the (-) enantiomer in rat brain membranes (Badio and Daly, 1994), so a slight lowering of the Hill number from unity, consistent with the slightly different binding affinities could be expected. The same argument could apply to anabasine, again the racemic form of the ligand used. However, this for instance, does not explain why (-)nicotine has a low Hill number.

4) Despite the inclusion of protease inhibitors in the isolation buffer to protect against protein degradation, it is possible that the membrane fractions were broken down as the experiments progressed. Leupeptin, pepstatin A and PMSF are well documented as inhibitors of mammalian proteases (see Merck Index). However, little is known regarding the nature and specificity of proteases in the insect nervous system. Indeed Lunt (1986) suspects that little effect is gained from the inclusion of anti-proteases in insect preparations. It is therefore possible that there was partial breakdown of the *Manduca* membrane preparations.

Muscarinic and mixed receptors in Manduca

Although, it has been established that the α -btx binding site in *Manduca* shows characteristic nicotinic pharmacology when compared with other insects, it was necessary to demonstrate that muscarinic compounds did not bind with high affinity. At 100 μ M concentrations, the majority of nicotinic ligands displaced nearly all specific [125 I] α -btx binding. In particular, nicotine, the definitive agonist of nAChRs inhibited ~85% of specific binding (Figure 3.7). In contrast, muscarine, the definitive agonist of mAChRs inhibited ~25% of specific binding in adults and ~65% of specific binding in larvae (Figure 3.8). These results agree with the conclusions previously made, that the α -btx binding site in *Manduca* is nicotinic, not muscarinic in nature, disputing Sanes *et al.* (1977) who suggested that it may be a mixed receptor. This does not refute the idea that there may be a mixed receptor of nicotinic and muscarinic pharmacology in the *Manduca* nervous system. Interestingly, Trimmer *et al.* (1993) have identified two [3 H]QNB binding sites, consistent with the presence of mAChRs in *Manduca*. The high affinity [3 H]QNB binding site has fewer binding sites ($B_{\max} = 23\text{fmol/mg protein}$) than the number of nicotinic binding sites obtained in this study, thus correlating with data from *Drosophila*.

In summary, these binding experiments demonstrate the presence of an α -btx sensitive nicotinic-like AChR in *Manduca* which is able to bind a variety of cholinergic ligands. Most importantly, nicotine binds with similar affinity in both adults and larvae as in other, nicotine sensitive insects, indicating that a naturally mutated binding site is improbable. Moreover, there do not appear to be α -btx insensitive binding sites that can be radiolabelled with [3 H](\pm)epibatidine. Although it is apparent that nicotine can bind to receptors in *Manduca*, without testing functional ability, it is impossible to tell whether binding of nicotine elicits an agonistic action on the receptor. An appropriate example is the effect of cytosine on rat $\alpha 4\beta 2$ receptors. Cytosine can bind with high affinity but acts only as a partial agonist at the ACh site (Papke and Heinemann, 1993; see Chapter 1,

section 1.2.5). The relationship between the binding data obtained in this chapter and the sequence data obtained in Chapter 2, is therefore the subject of Chapter 4.

CHAPTER 4

An overall perspective

4.1 Relating sequence and binding data

This study has resulted in the isolation of two overlapping clones encoding a putative α -like subunit of the nAChR (Chapter 2), and the characterisation of a membrane bound α -btx binding component from *Manduca* larvae and adults with a profile typical of a nAChR (Chapter 3). The affinity of nicotine for this α -btx binding site was comparable with values described in the literature for other nicotine sensitive insects, indicating that the nicotine resistance intrinsic to *Manduca* was not as a result of a modified α -btx binding receptor (see Chapter 3). This was compatible with the sequence data obtained from the two overlapping clones, analysis of which implied that all the residues known to be essential for ligand binding, and in particular nicotine binding, were present, suggesting that nicotine resistance in *Manduca* was not due to a modified α -like subunit of the nAChR (see Chapter 2). This does not prevent the possibility that nicotine acts as a partial agonist (see Chapter 3, section 3.5) where nicotine binds with high affinity to the nAChR but has low efficacy in evoking responses.

Although a modified nAChR may not be responsible for *Manduca*'s nicotine resistance, there remains the possibility that fewer binding sites are available with which nicotine can interact compared with other nicotine sensitive insects. This hypothesis is hampered by lack of evidence from the larval forms of other insects, so although the number of α -btx binding sites (and therefore potential nicotine binding sites) appears low (119 fmol/mg protein) in comparison with adults (815 fmol/mg protein) (see Chapter 3), this may be the case for all larval forms from other related species.

Interestingly, data obtained from the inhibition of specific radiolabelled α -btx binding by various cholinergic ligands fitted a single binding site model, suggesting that there was only one class of binding site. Furthermore, the use of a radioligand (radiolabelled epibatidine) that in vertebrates has been shown to label sites distinct from those labelled by α -btx (see Chapter 3), hinted at the sole presence of α -btx binding receptors. Again, this is consistent with the isolation of a single α -like subunit. Despite numerous attempts, further α -like subunits were not isolated, tentatively suggesting that only one α -like subunit exists in *Manduca*. Moreover, this agrees with the fact that only one (partial length) cDNA clone encoding a putative β -like subunit has been isolated from *Manduca*, MARB1 (J. Eastlake, University of Bath). It can therefore be speculated that the presence of a single α -like subunit and the presence of a single β -like subunit is in accord with an heteromeric receptor, comprising two identical α -like subunits and three identical β -like subunits, assuming a stoichiometry equivalent to that postulated for

vertebrates (Anand *et al.*, 1991; Cooper *et al.*, 1991). As the 'ligand binding' subunits would be surrounded by identical 'structural' subunits only one high affinity binding site might be expected, consistent with the data obtained on *Manduca* membranes.

This single high affinity binding site model caused by identical α -like and identical β -like subunits may be similar to the native $\alpha 7$ receptor that binds α -btx and is postulated to be heteromeric (Couturier *et al.*, 1990b; Anand *et al.*, 1993). This model however, does not compare well with other insect data. For example, the *Drosophila* subunit ALS formed functional receptors in *Xenopus* oocytes only when it was expressed with the chick $\beta 2$ subunit (Bertrand *et al.*, 1994), ACh evoked responses being blocked by low concentrations of α -btx. However, ARD was known to be associated with ALS *in vivo* (Schloss *et al.*, 1991) but did not form functional receptors with ALS in *Xenopus* oocytes (Bertrand *et al.*, 1994) suggesting that another non-identical subunit was necessary for the formation of the native receptor. This would probably mean the presence of more than one binding site for this receptor, consistent with data obtained by Schloss *et al.* (1988) which indicated the presence of two [125 I] α -btx binding sites. On the other hand, Sgard *et al.* (1993) found evidence of only one α -like subunit for the lepidopterans, *Chilo suppressalis*, *Heliothis virescens* and *Spodoptera frugiperda* (see Chapter 1, section 1.3.2). However, there is neither α -btx binding data nor data concerning the search for β -like subunits available to support this finding.

Binding data using radiolabelled α -btx was obtained for two developmental stages, larvae and adults in the life cycle of *Manduca*. Statistical analysis suggested that there was no difference in the binding of nicotine between these two developmental stages. This implied that the same class of α -btx binding site was present in both developmental stages. It was vital to establish this point, as larvae and adults have different feeding habits (see Chapter 1, section 1.1.2): tobacco chewing larvae would require mandatory protective mechanisms against nicotine poisoning whereas in adults this is not necessary due to feeding on nectar. The presence of equivalent α -btx binding sites is once again consistent with the analysis of sequence data. The two overlapping clones were isolated from embryo and adults and would therefore develop into nicotine ingesting larvae and non-nicotine ingesting adults respectively. The presence of two different α -like subunits corresponding to the two stages of the life cycle would result in two different sequences. However, the overlapping region (within the extracellular region of the subunit) of the two clones was identical in both nucleotide and amino acid sequence, consistent with the presence of a single type of receptor. Interestingly, electrophysiological studies on the PPR motoneuron in the prepupal stage of *Manduca* development demonstrated

resistance to nicotine (Trimmer and Weeks, 1989). The authors suggested that further investigations on the accessory planta retractor motoneuron, which receives a mono-synaptic afferent input, is cholinoreceptive, and unlike the larval PPR, survives to adult eclosion could be used for developmental studies of nicotine resistance. This would therefore be an ideal system for demonstrating resistance to nicotine in both nicotine ingesting larvae and adults at the individual neuron level.

4.2 Future directions

Sequence comparison with other sequences encoding insect α -like subunits of the nAChR strongly suggests that MARA1 would also form an α -like subunit of the nAChR. However ultimate proof will only be attained through expression of the full-length MARA1 and functional analysis, for example, in *Xenopus* oocytes. Two options are possible: 1) alone MARA1 can form functional receptors, indicating that it has the ability to form homomers or 2) alone MARA1 cannot form functional receptors, indicating that other subunits are imperative for correct assembly. In the latter case, co-injection of the cDNA encoding MARA1 with the cDNA encoding the putative β -like subunit from *Manduca*, MARB1 (once obtained in its full-length form) would indicate whether a functional heteromeric receptor is formed. However, as previously shown with the *Drosophila* subunits ALS and ARD, co-expression of two subunits in *Xenopus* oocytes is not always definitive of their ability to combine in the same receptor (Bertrand *et al.*, 1994).

Although it is apparent that nicotine can bind to receptors in *Manduca* and that residues involved in nicotine binding are present in MARA1, without testing functional ability, it is impossible to tell whether binding of nicotine elicits an agonistic action on the receptor. An appropriate example is the effect of cytosine on rat $\alpha 4\beta 2$ receptors. Cytosine can bind with high affinity but acts only as a partial agonist at the ACh site (Papke and Heinemann, 1993) and the same may be true of nicotine at nAChRs in the *Manduca* nervous system. Even so, nicotine may still be toxic, by acting as an antagonist and preventing access of ACh to the nAChR.

The possible co-expression of MARA1 and MARB1 could also be investigated using immunoprecipitation experiments in a similar manner to that for the *Drosophila* ALS and ARD subunits (Schloss *et al.*, 1991). Moreover, such experiments could relate to the α -btx binding sites present in *Manduca* nervous system membranes. Antisera against bacterially expressed fusion proteins that included the sequence of the cytoplasmic loop for each putative subunit and hence were subunit specific could be raised and used to

immunoprecipitate [125 I] α -btx binding sites solubilised from *Manduca* membrane preparations. If antisera to both MARA1 and MARB1 precipitated all the α -btx binding sites, it would be further indication that both subunits formed part of the same receptor and that there was only one class of α -btx binding site. Alternatively, if either one precipitated a different proportion of sites than the other, it would imply that additional combinations of subunits existed in the *Manduca* nervous system rather than the single combination postulated by this study.

In the same vein, subunit specific antibodies directed against the cytoplasmic loops of MARA1 and MARB1 or fluorescein isothiocyanate labelled α -btx in conjunction with confocal laser scanning microscopy (Wheeler *et al.*, 1994) could be used to localise receptors within the *Manduca* nervous system. Moreover, *in situ* hybridisation experiments could show where transcripts of these putative subunits were expressed. These studies would be ideal for observing the presence of MARA1 and MARB1 throughout the different stages of development and could be correlated with the autoradiographic localisation of α -btx binding sites presented by Hildebrand *et al.* (1979).

4.3 Why is *Manduca* resistant to nicotine?

Having virtually eliminated the possibility of a modified nAChR as being the mechanism of nicotine resistance in *Manduca*, it is necessary to ask again, why is *Manduca* resistant to nicotine, when nicotine if eaten by other insects proves so fatal? Once again, it is necessary to allude to *Manduca*'s ability to ingest not only nicotine but other alkaloids (see Chapter 1, section 1.1.2) that would be deadly for other insects. This suggests at the very least, a basic common mechanism for protection against harmful allelochemicals.

The following mechanism of protection against the toxic effects of nicotine and other alkaloids is an amalgamation of hypotheses from various sources. However, comprehensive comparison with another lepidopteran that is susceptible to nicotine (and other alkaloid) poisoning is necessary to ensure that the biological machinery used in *Manduca* is not commonplace to all lepidopterans.

Rapid metabolism of nicotine in the midgut of the larvae to more polar metabolites aids excretion and thus is the first protective mechanism against nicotine toxicity (Snyder *et al.*, 1994). Moreover, nicotine induces its own metabolism in the midgut (Snyder *et al.*, 1993) by switching on various cytochrome P-450 mono-oxygenase activities. It is

possible that different alkaloids activate different subsets of these enzymes and therefore offer a protective mechanism unique to a particular alkaloid (see Chapter 1, section 1.1.3, Metabolism and excretion in *Manduca*).

Although this accounts for most of the absorbed nicotine, it does not explain why the CNS of *Manduca* is intrinsically insensitive to nicotine while remaining sensitive to other cholinergic agents (Morris, 1984; Trimmer and Weeks, 1989). The rapid metabolism of nicotine by the CNS has been observed (Morris, 1983a, Snyder *et al.*, 1993) in contrast to results obtained in this study using inhibition of an α -btb binding site (presumably an nAChR) by nicotine (see Chapter 3). Considering that the CNS of *Periplaneta*, a nicotine sensitive insect, was also shown to metabolise nicotine (Morris, 1983a) it is unlikely that metabolism in the CNS is the sole protective mechanism of the CNS.

It has been postulated that the blood-brain barrier is an effective mechanism against nicotine penetration to nAChRs in the neuropile (Morris and Harrison, 1984). This is due to the lack of cell to cell contacts in the basal perineurial layer which preclude the penetration of protonated nicotine, in contrast to the many cell to cell contacts seen in the basal perineurial layer of the nicotine sensitive insect, *Periplaneta*. These observations would explain the global electrophysiological recordings obtained on *Manduca* and *Periplaneta* by Morris (1984) which demonstrated that the intact nerve cord of *Manduca* was less sensitive to nicotine than the intact nerve cord of *Periplaneta*.

However, studies on desheathed nerve cords (Morris, 1984) and on a desheathed isolated motoneuron (Trimmer and Weeks, 1989) from *Manduca* exhibited a similar result with nicotine: although sensitivity had increased, it was not as equally responsive as *Periplaneta* nerve cords (David and Sattelle, 1984). Other cholinergic ligands induced neural activity of similar effectiveness in both species, indicating that this phenomenon was peculiar to nicotine (of the ligands tested; see Chapter 1, Table 1.1).

Ultrastructural studies on the nerve cords of *Manduca* have revealed that desheathing causes only localised damage (Morris and Harrison, 1984). It was demonstrated that despite the removal of the neural lamella and outer perineurial cells, the interface of the perineurium with the underlying glial/neuronal mass was intact and the areas adjacent to the desheathed perineurium retained their integrity. Furthermore, ionic lanthanum (a cation, presumed to penetrate the blood-brain barrier to the same extent as cationic nicotine) was only partly successful in penetrating the desheathed nerve cords. It appeared that lanthanum gained access to the cytoplasm of damaged cells and also the intercellular clefts where perineurial cells were relatively intact but did not penetrate even

the most peripheral regions of the subperineurial extracellular space. As nAChRs would be located at the junction of the inner regions of the extracellular space with the neuropile, it is likely that desheathing of the nerve cords does not grant access to the nAChRs and thus results in the low sensitivity seen with nicotine, as in its protonated form it is unable to interact in a sufficient amount with the receptor. In this context, it is interesting that Trimmer and Weeks (1989) "took great care in removing as much of the sheath as possible", however, as they did not perform ultrastructural studies to assess the damage caused by desheathing, the probability of nicotine reaching the neuropile and thus interacting with the nAChR is not known.

In insects, generally nicotine can reach the synapse and interact with the nAChR if the lipophilic form can penetrate the integument and the ion and blood-brain barriers to establish an equilibrium where protonated nicotine is formed at the synapse (Figure 4.1; see Chapter 1, section 1.1.3, Barriers to nicotine penetration).

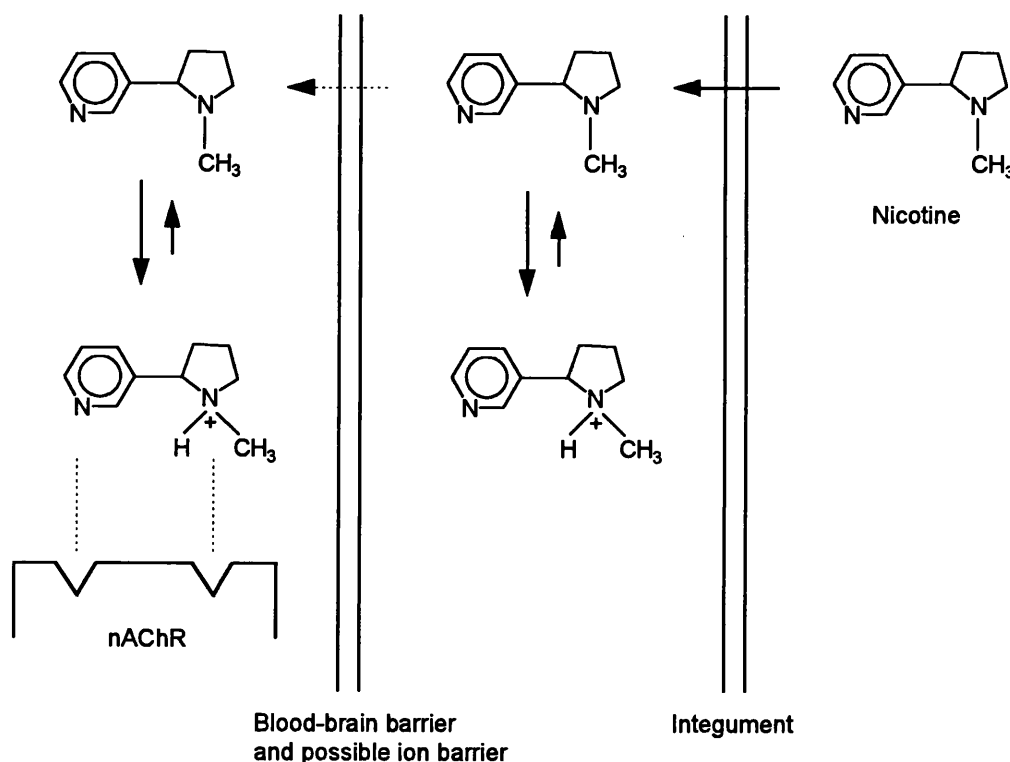


Figure 4.1. The route taken by nicotine to nAChRs in the CNS of insects when used as an insecticide. Dashed lines indicate interaction of nicotine with the nAChR. Dashed arrows represent minor routes. Re-drawn from Yamamoto *et al.* (1995).

Considering that the pH of the haemolymph in *Manduca* larvae is 6.6, there is little nicotine present in the lipophilic form (Self *et al.*, 1964a) to penetrate any barriers and as the blood-brain barrier has few cell-to-cell contacts, there are few access points left for the penetration of protonated nicotine. Furthermore, lipophilic nicotine entering the cells of the blood-brain barrier may be metabolised by the local detoxification system of the cells. Consequently, it is possible that hardly any nicotine reaches the synapse in either lipophilic or protonated form and therefore *Manduca* is not poisoned by the nicotine entering its system.

In summary, a combination of factors may account for *Manduca*'s resistance towards nicotine:

- Metabolism of nicotine in the midgut to more polar metabolites.
- Rapid excretion of the metabolic products by an active transport system.
- Possible metabolism in the CNS of the remaining lipophilic form of nicotine.
- Effective blood-brain barrier preventing access to the synapse of the protonated form of nicotine.

The end result: very little nicotine reaches the synapse and interacts with the nAChRs. Consequently, a depolarising blockade is not reached and *Manduca* is not killed. A combination of experiments would be necessary to test this hypothesis: the specific cytochrome P450 mono-oxygenase activities could be inhibited to test for lack of metabolism in the midgut and CNS while comparison of nicotine penetration into intact and completely desheathed neurons (as determined by ultrastructural studies) could test for penetration through the blood-brain barrier.

CHAPTER 5

Two electrode voltage clamp recording from *Xenopus* oocytes

5.1 Introduction

The *Xenopus* oocyte expression system in conjunction with two electrode voltage clamp recording was used primarily as a training exercise with a view to expressing the *Manduca* α -like subunit if a full length clone was isolated. Mouse muscle nAChR was characterised using the nicotinic agonist anatoxin (together with three of its analogues) and the nicotinic antagonist methyllycaconitine.

5.1.1 Two electrode voltage clamp

Voltage clamp is a means of controlling the potential across a membrane. In a voltage clamp experiment, a voltage is applied across the membrane and the current across the membrane that is required to maintain that particular voltage is measured. Early studies used the squid giant axon to develop this technique (Hodgkin and Huxley, 1952). Voltage clamp is more commonly used than the alternative current clamp (in which the current is clamped and the voltage recorded) as firstly, it eliminates capacitive current so that only ionic current is measured; secondly, the currents that flow are proportional to the membrane conductance, i.e. the number of open channels, and thirdly, it offers tight control over opening and closing of ion channels if they are voltage gated.

Xenopus oocytes offer a convenient lipid membrane in which ion channels can be expressed and their characteristics determined by voltage clamp. In single electrode voltage clamp, the same electrode clamps the voltage and records the current. This study however, utilises two electrode voltage clamp whereby one electrode maintains the voltage at a set potential and the other measures the current across the membrane. Macroscopic currents from the whole cell (which is expressing hundreds of ion channels correlating with the exogenous genetic material injected - see section 5.1.2) are recorded. This is somewhat different from the recording of single channel currents by patch clamping.

In the case of currents elicited by LGICs, such as those of the nAChR, ion flux is only observed on application of the appropriate agonist to the oocyte, once any leakage has been eliminated. Thus, in a voltage clamp experiment, once the nAChRs of an oocyte resting in a bath of physiological buffer and clamped at a negative holding potential have been activated by the perfusion of agonist into the bath, cations such as Na^+ flow inward through the ion channels, causing the current to change in order to maintain the voltage at its holding potential and this is seen, by convention, as a downward deflection of the current trace.

5.1.2 The *Xenopus* oocyte expression system

Oocytes (germ cells) from the South African clawed frog, *Xenopus laevis* are commonly used as a model system for the expression of ion channels as they are able to synthesise exogenous proteins when injected with foreign mRNA into the cytoplasm (Gurdon *et al.*, 1971) or cDNA into the nucleus (Ballivet *et al.*, 1988). Moreover, they are capable of post-translational modifications such as phosphorylation and glycosylation, as well as the correct assembly and insertion of subunits into the surface membrane (reviewed in Kushner *et al.*, 1989).

Generally, ion channel expression studies use fully grown immature oocytes which due to their large size can be easily manipulated. The fully grown oocyte (stage 6) is between 1 and 1.3mm in diameter and has a characteristic appearance, being divided into hemispheres of the animal (dark brown) and vegetal (yellow) poles, as shown in Figure 5.1. The darker colour of the former is due to the higher concentration of melanin containing granules. The oocyte is surrounded by several layers: a vitelline membrane which is non-cellular and fibrous; a follicle cell layer which possesses numerous gap junctions forming contacts with the oocyte; the theca which is a connective tissue layer and finally an epithelial cell layer which is a continuation of the ovary wall. The outer layers contain the oocyte's endogenous β -adrenergic, muscarinic and purinergic receptors and therefore are removed either mechanically or enzymatically with collagenase (reviewed in Dascal, 1987). The innermost vitelline membrane is removed mechanically for patch clamp recording only (Methfessel *et al.*, 1986).

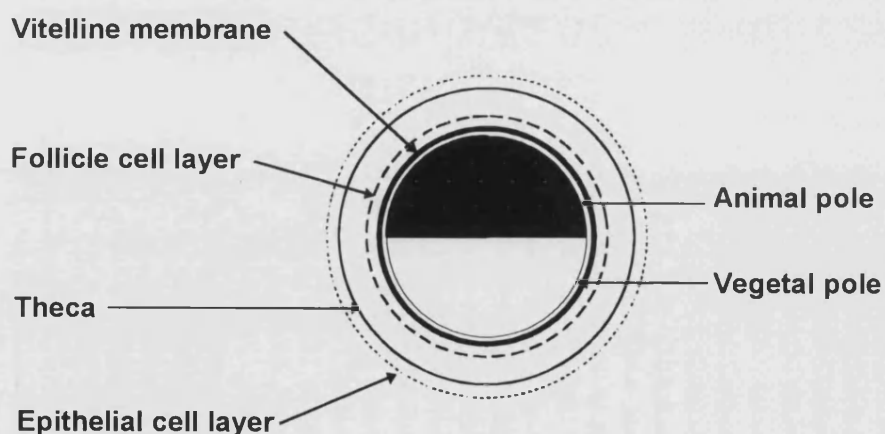


Figure 5.1. Schematic of a *Xenopus* oocyte. Adapted from The Axon Guide for Electrophysiology and Biophysics Laboratory Techniques (1993).

Following the injection of foreign genetic material, the oocyte expression system aids studies of ion channels due to the following reasons: a) the high proportion of injected cells expressing the functional protein, b) the ease of manipulation of the environment surrounding the oocyte by bath perfusion of drugs or ions and c) the control of the membrane potential of the oocyte by voltage clamp. However, there are some disadvantages to the oocyte expression system. These include the short expression period compared with stably transfected cell lines (at most, two weeks of expression before oocyte deterioration); the small number of oocytes that can be assessed for function during one experiment (resulting in the necessity for a normalised response for each oocyte) and the seasonal variation in the quality of the oocytes. In addition, if *in vitro* transcription of cDNA is used to produce mRNA for injection into the oocyte, extreme care is required to prevent degradation by RNases (Sigel, 1990).

5.1.3 The muscle nAChR

The ability of oocytes to form functional nAChRs was originally demonstrated by the presence of specific [¹²⁵I]α-btx binding sites following injection of mRNA from *Torpedo* electroplax (Sumikawa *et al.*, 1981) whose ion channels were later shown to be gated by ACh (Barnard *et al.*, 1982). cDNA clones encoding putative α, β, γ and δ subunits of the *Torpedo californica* nAChR (Noda *et al.*, 1982, 1983a,b) were shown to form a functional receptor when expressed together in *Xenopus* oocytes (Mishina *et al.*, 1984). Results from the deletion of each subunit suggested that all four subunits were required to elicit an ACh response characteristic of a nAChR whereas only the α subunit was imperative for α-btx binding activity. Moreover, it was found that fetal and adult nAChRs in calf muscle express ion channels that differed in their conductance and gating properties due to the replacement of the γ subunit in the fetal receptor with the ε subunit in the adult receptor (Mishina *et al.*, 1986). The expressed receptors had properties that were similar to *in situ* receptors. Homology of the individual subunits among a range of organisms was high and it was demonstrated that chimeric receptors consisting of mRNA encoding mixed subunits from *Torpedo* and either calf or cat formed functional receptors in oocytes, the properties of which depended on the particular subunits chosen for expression (Sakmann *et al.*, 1985; Sumikawa and Miledi, 1989).

It was later demonstrated that oligomerisation and functional nAChR formation in oocytes did not require injection of all four subunit RNAs (White *et al.*, 1985; Kurosaki *et al.*, 1987) although nAChRs that were devoid of a subunit functioned to a lesser extent than those comprising all four subunits. However, Buller and White (1990a) discovered that

Xenopus oocytes express low levels of nAChR α subunit mRNA that, when translated could lead to the formation of functional *Xenopus-Torpedo* nAChR hybrids.

5.1.4 Anatoxin and methyllycaconitine

Anatoxin-a

Anatoxin-a (AnTx) is a potent nicotinic agonist produced by the freshwater filamentous cyanobacterium, *Anabaena flos aquae*. Surface blooms of *Anabaena* have killed livestock and waterfowl by the creation of a depolarising blockade at muscle-type nAChRs (as described in Chapter 1, section 1.1.1) and subsequent respiratory paralysis (Carmichael *et al.*, 1975). The physiological effects of AnTx are very similar to those of ACh. Single channel patch clamp recordings on frog muscle fibres have shown that both agonists activate channels with virtually identical conductances ((+)AnTx: 28pS; ACh: 27pS) although (+)AnTx binds with higher affinity at the [¹²⁵I] α -btx binding site on *Torpedo* electroplax membranes than ACh and elicits contracture of frog muscles at lower concentrations than ACh (after AChE inhibition), indicating that it is a more potent agonist than ACh (Swanson *et al.*, 1986). In contrast, (-)AnTx is less potent than ACh (Swanson *et al.*, 1986) indicating that AnTx is stereospecific in its agonistic effects. The (+) isomer is the natural form of AnTx.

In rat brain, competition binding assays have shown that AnTx binds to the high affinity [³H]nicotine binding site (MacAllan *et al.*, 1988), which correlates with the $\alpha 4\beta 2$ subtype (Whiting and Lindstrom, 1986b; Flores *et al.*, 1992; see Chapter 3, section 3.1.1) and to the [¹²⁵I] α -btx binding site (MacAllan *et al.*, 1988) which correlates with the $\alpha 7$ subtype in chick (and presumably in rat also) (Schoepfer *et al.*, 1990; see Chapter 3, section 3.1.1) although selectivity is shown toward the [³H]nicotine binding site. Furthermore, Thomas *et al.* (1993) have shown that (+)AnTx is approximately 200-fold more potent than ACh in functional assays of $\alpha 7$ receptors expressed in *Xenopus* oocytes, making it, along with epibatidine (Badio and Daly, 1994), one of the most effective nicotinic agonists known.

Anatoxin-a analogues

AnTx is a secondary amine (with the nitrogen in a bicyclic ring system) complete with a conjugated enone as shown in Figure 5.3. The rigid structure of the azabicyclononene ring of AnTx, its small size (molecular weight 166), the high stereoselectivity for the (+) enantiomer and the available synthetic chemistry make AnTx an ideal parent compound for structure activity studies. Analogues based on AnTx can help to define the agonist recognition site and thus have been synthesised with systematic modifications and assayed for agonist potency using binding assays and electrophysiological recordings.

Using a series of AnTx analogues with modifications of the acetyl side chain, Swanson *et al.* (1991) and Wonnacott *et al.* (1991) found that all additions to the acetyl side chain resulted in a loss of agonist potency for the muscle nAChR as determined by frog muscle contracture assays and [125 I] α -btX binding assays and the neuronal nAChR as determined by [3 H]nicotine and [125 I] α -btX binding assays to rat brain membranes. In the latter case, all the analogues had higher affinity for the [3 H]nicotine binding site than the [125 I] α -btX binding site but the rank order of potency remained the same at both sites and furthermore, agreed with the rank order of potency at the muscle [125 I] α -btX binding site (Wonnacott *et al.*, 1991). More recently, three new analogues which have an increasing number of methine or methylene units added to the acetyl side chain of AnTx have been synthesised (Thomas *et al.*, 1994). These are homoanatoxin (HAnTx), propylanatoxin (PAnTx) and isopropylanatoxin (IPAnTx) and are shown in Figure 5.3. Competition binding assays using [3 H]nicotine and [125 I] α -btX at the neuronal nAChR indicated that the analogues retained or exceeded the potency of (\pm)AnTx, although PAnTx showed marked preference for the [125 I] α -btX binding site (Thomas *et al.*, 1994). Similarly, [125 I] α -btX binding assays on mouse skeletal muscle showed that the analogues exceeded the potency of the parent compound (Thomas, 1996). However, another study demonstrated that HAnTx possessed one tenth of the activity of (+)AnTx in frog muscle contracture assays (Wonnacott *et al.*, 1992) and functional potency values for $\alpha 7$ receptors expressed in *Xenopus* oocytes showed that (\pm)AnTx was more potent than either (\pm)HAnTx or (\pm)IPAnTx (PAnTx not tested) (Thomas *et al.*, 1994).

Methyllycaconitine

Methyllycaconitine (MLA) is a nicotinic antagonist found in the seeds of *Delphinium brownii*. Initial studies demonstrated that MLA blocked the neuromuscular junction in the rat phrenic nerve-diaphragm preparation with an ED₅₀ in the micromolar range (Nambi-Aiyar *et al.*, 1979) which agreed with binding affinities obtained from MLA displacement of [125 I] α -btX binding in *Torpedo* electroplax and frog and human muscle preparations (MacAllan *et al.*, 1988; Ward *et al.*, 1990). In the rat brain, MLA was more effective at inhibiting binding to the [125 I] α -btX binding site than to the [3 H]nicotine binding site (MacAllan *et al.*, 1988) and in addition was more effective at the [125 I] α -btX site in brain than in muscle (MacAllan *et al.*, 1988; Ward *et al.*, 1990), suggesting that MLA is a selective toxin for the former site. This finding was reinforced by expression of neuronal nAChRs in *Xenopus* oocytes. Although ACh evoked responses were blocked by MLA in $\alpha 3\beta 2$, $\alpha 4\beta 2$ and $\alpha 7$ receptors, MLA displayed a 3000-fold preference for the $\alpha 7$ receptor over the $\alpha 3\beta 2$ receptor and a 26000-fold preference for the $\alpha 7$ receptor over the $\alpha 4\beta 2$ receptor (Drasdo *et al.*, 1992; Wonnacott *et al.*, 1993; Palma *et al.*, 1996).

5.1.5 Aims

The aims of this study therefore, were to prepare mRNA encoding mouse muscle nAChR subunits by *in vitro* transcription, to micro-inject this mRNA into denuded oocytes and to characterise the functional receptors formed by analysis of two electrode voltage clamp recording when the receptors were activated by AnTx and analogues using ACh as a standard or when ACh evoked responses were blocked by MLA.

These ligands were chosen as their effects on muscle nAChR as expressed in *Xenopus* oocytes had not been previously characterised. Furthermore, this study was performed with a view to expressing the *Manduca* α -like subunit MARA1, if a full length clone was isolated. In this case, a range of ligands would be studied in order to analyse their effects on the agonist binding site. The inclusion of AnTx, its analogues and MLA would be appropriate as AnTx shows selectivity for the [^3H]nicotine binding site (which in *Manduca* may not be present; see Chapter 3) whereas MLA shows high selectivity for the [^{125}I] α -btx binding site which is present in *Manduca* and is displaced by low concentrations of MLA (see Chapter 3).

5.2 Materials

AnTx, HAnTx, PAnTx and IPAnTx were synthesised by Professor T. Gallagher (School of Chemistry, University of Bristol, Bristol, U.K.) as described in Thomas *et al.* (1994). MLA was isolated from *Delphinium* Garden Hybrid seeds according to the method of Coates *et al.* (1994) by Dr I S Blagborough and Professor B V L Potter (School of Pharmacy and Pharmacology, University of Bath, Bath, U.K.). cDNAs encoding mouse muscle subunits were provided by Dr J Boulter (Salk Institute, California, U.S.A.). Mature female *Xenopus* were purchased from Blades Biologicals, Edenbridge, U.K.

DNA restriction enzymes and RNA modifying enzymes were supplied by New England BioLabs, Hitchin, U.K. and Boehringer Mannheim U.K. Ltd., Lewes, U.K. respectively. All other reagents were from the Sigma-Aldrich Chemical Company Ltd., Poole, U.K. and were of Analar grade.

Drummond glass capillaries were obtained from Laser Laboratory Systems Ltd., Southampton, U.K. while borosilicate glass capillaries were obtained from Clark Electromedical Instruments, Reading, U.K. Falcon plasticware was purchased from Becton Dickinson and Company, Plymouth, U.K.

AnTx and analogues were made up as 20mM stock solutions in 19% ethanol whereas MLA was made up as a 20mM stock solution in 100% ethanol. Subsequent concentrations of drugs were prepared in standard oocyte saline (see section 5.3).

5.3 Methods

5.3.1 *In vitro* transcription

Plasmid DNA encoding mouse muscle nAChR subunits was prepared from glycerol stocks as described in section 2.3.1. The DNA encoding each subunit (10µg) was linearised with the appropriate restriction enzyme (α , β and δ with *HindIII*, γ with *EcoRI*) at 37°C overnight as described in section 2.3.4. The resulting linearised template was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) before being precipitated with 0.1 volumes sterile 3M sodium acetate and 3 volumes sterile absolute ethanol at -20°C overnight. Linearised template was centrifuged at high speed for 30min, washed with sterile 70% ethanol, dried and resuspended in 20µl RNase free DEPC treated sterile water.

Maintaining RNase free conditions, 5µl 10x transcription buffer (0.4M Tris-HCl pH8.0, 60mM MgCl₂, 100mM DTT, 20mM spermidine), 5µl each of 100mM DTT, 5mM ATP, 5mM CTP and 5mM UTP, 5µl 0.5mM GTP, 0.5µl 10mg/ml BSA, 2.5µl cap analogue (m⁷G(5')ppp(5')G), 10µl linearised template DNA, 2µl 40U/µl RNase inhibitor and 2µl SP6 polymerase in a total volume of 50µl were mixed and incubated at 40°C for 1h. The resulting transcripts were precipitated with 0.1 volumes 10M ammonium acetate and 3 volumes absolute ethanol at -70°C until required for injection. At this point, appropriate volumes (approximately 10-20µl) of transcript from each subunit were mixed, centrifuged at high speed for 20min, washed in 70% ethanol, dried and resuspended in 5-10µl (depending on average concentration of transcripts) of sterile RNase free water.

To assess the quality and quantity of the transcripts, a parallel but smaller transcription was set up incorporating [α -³²P]UTP. In this, all reagents were scaled down, 1µl of [α -³²P]UTP was added and a 20µl total volume was obtained.

The quality of RNA produced from *in vitro* transcription of RNA was analysed on a denaturing gel as described in section 2.3.3. The quantity of RNA was analysed by a DE-81 filter binding assay. In this, 2µl of the radioactive transcription mixture was mixed with 18µl of RNase free water. To each of two DE-81 filters was added 8µl of the diluted transcription mixture. One filter only was washed 4 times in 30ml 0.5M dibasic sodium phosphate buffer and then twice in water and once in absolute ethanol. Both filters were air dried, added to 5ml 'Optiphase Safe' scintillant and counted on a Packard Tricarb

1600 TR scintillation spectrometer. The amount of transcript was then calculated using the amount of radioactivity incorporated into the synthesised transcript.

5.3.2 *Xenopus* and *Xenopus* oocytes

***Xenopus* maintenance**

Female *Xenopus laevis* were maintained in colonies of 18 animals in chlorine free water under conditions of 18-22°C and 14:10 h light:dark ratio. Each colony was fed with minced heart twice weekly and each tank was cleaned out twice weekly.

Preparation of oocytes

Technical assistance was provided by members of the Animal House at the University of Bath who held a Home Office licence for the following procedure. *Xenopus laevis* were anaesthetised by submersion in 0.15% Tricaine (3-aminobenzoic acid ethyl ester) at room temperature for approximately 40min until leg retraction ceased. Ovarian lobes were extracted through an incision made in the skin and abdominal body wall in the posterior ventral side of the frog. The incision was sutured using Mersilk™ 4.0 (Ethicon Ltd., U.K.) and the frog was left to recover in water before returning to its original colony. The ovarian lobes were sliced into smaller clumps of approximately 50 oocytes and the follicle cell and thecal layers removed from the oocytes by incubation with collagenase (type 1A; 2mg/ml) in sterile standard oocyte saline (SOS: 100mM NaCl, 2mM KCl, 1.8mM CaCl₂, 1mM MgCl₂, 5mM HEPES, pH7.6) at 18°C with rotation for 1.5-2h. Separated oocytes were rinsed in several washes of SOS and stored for up to 3 days at 4°C. Oocytes of the appropriate size and with well defined poles were selected from microscopic viewing.

Injection needles were pulled from 3.5" Drummond glass capillary tubing on a moving coil microelectrode puller (Campden Instruments Ltd.) to produce a tip of approximately 1µm which was then gently broken to give a tip with a slightly larger diameter. Needles were sterilised by baking at 200°C for at least 2h before being back filled with sterile mineral oil and attached to the plunger of a Drummond micropipettor (Laser Laboratory Systems Ltd.). The needles were filled with samples by manual control of the micromanipulator that was attached to the micropipettor. With the aid of a binocular microscope and an automatic 'Nanoject' microinjector, oocytes were co-injected with at least 50ng of combined *in vitro* transcripts in a total volume of 50nl. Oocytes were incubated at 18°C for 1 to 7 days in changes of SOS supplemented with 10U/ml penicillin, 10µg/ml streptomycin and 50µg/ml gentamycin. Control oocytes were injected with sterile water and incubated under the same conditions.

5.3.3 Two electrode voltage clamp

Microelectrodes were pulled from borosilicate glass capillaries on a moving coil microelectrode puller (Campden Instruments Ltd.), slightly bent in a bunsen flame and back filled with 3M KCl (pH 7.8). Each oocyte was positioned in the recording chamber and impaled with two microelectrodes, each with a resistance less than 1m Ω . Electrophysiological recording was performed using a CA 100 clamp amplifier (Biologic, France) on oocytes with a resting membrane potential of greater than -20mV. Oocytes were perfused with SOS supplemented with 1 μ M atropine (to prevent activation of any remaining endogenous mAChRs) at a rate of 12ml/min and voltage clamped at -80mV unless otherwise stated. The desired dose of agonist was applied for 5s by perfusion through a pinch valve system. For antagonist experiments, oocytes were incubated in MLA for 5min before application of ACh in the presence of MLA. Control perfusions used the highest dose of ethanol that would be found in a drug application. In each case, the change in current (or lack of) across the membrane of the oocyte required to keep the oocyte clamped at -80mV was recorded on an oscilloscope and chart recorder.

Data was stored on a digital-to-analogue DAT converter (Biologic) for later analysis. Data was acquired using an AT-MIO-16D expansion board (National Instruments) and analysed using the program Daqware. Data was plotted using the program Sigmaplot (Jandel Scientific). Dose response curves for the agonists were fitted to the non-linear Hill equation, $y = 100\%/[1+(EC_{50}/[ligand])^{nH}]$ where y is the normalised current (%), EC_{50} is the concentration of ligand that evokes 50% of the normal response, [ligand] is the agonist concentration and nH is the Hill number. Dose response curves for MLA were fitted to the non-linear Hill equation, $y = 100\%/[1+([ligand]/IC_{50})^{nH}]$ where y is the normalised current (%), [ligand] is the antagonist concentration, IC_{50} is the concentration of the competing ligand that inhibits 50% of the normal response and nH is the Hill number.

5.4 Results

In vitro transcriptions of the mouse muscle subunits were generally successful, as shown in Figure 5.2. Measurement of the amount of transcript resulted in concentrations of approximately 200ng/ μ l for each transcript.

Two electrode voltage clamp recording was used to assess the effects of agonist and antagonist application on mouse muscle nAChRs expressed in *Xenopus* oocytes. Preliminary experiments demonstrated that application of ACh for 5s, 10s or 20s resulted in currents with the same amplitude as the nAChRs desensitised within the first 5s of the drug application (data not shown). An assessment of the agonist potencies of AnTx, HAnTx, PAnTx and IPAnTx as compared with ACh used increasing drug concentrations over the range 0.1 μ M to 100 μ M. Application of agonist resulted in a large inward current of up to 2.5 μ A which began to desensitise within 5s of agonist application. The greater the drug concentration, the greater the size of the current. At the highest concentration tested, ACh and AnTx appeared to be equi-efficacious whereas the three AnTx analogues gave somewhat smaller currents (Figure 5.3).

Dose response curves (Figure 5.4) generated the following EC₅₀ values: AnTx 8.2 \pm 2.7 μ M, HAnTx 71 \pm 6.9 μ M, PAnTx 80 \pm 4 μ M and IPAnTx 72 \pm 17 μ M. In comparison, ACh gave an EC₅₀ value of 33 \pm 4.3 μ M. This resulted in a rank order of potency AnTx > ACh > HAnTx = IPAnTx > PAnTx. The three analogues did not attain peak currents corresponding to an 100% response as the concentrations necessary to achieve this were too high.

Neither water injected controls nor perfusion of ethanol corresponding to the highest concentration found in drug solutions resulted in change of current.

Current-voltage relationships were determined for AnTx (50 μ M) and ACh (50 μ M) (Figure 5.4). ACh evoked responses decreased in size as the membrane potential was increased step-wise from -100mV to 0mV and then increased in size and direction corresponding to a change to positive potentials. However, AnTx evoked responses appeared to show a non-linear decrease with increasingly positive potentials, until no responses were observed at positive potentials appearing to exhibit inward rectification.

ACh evoked responses were fully blocked by 10 μ M MLA. Application of MLA to the oocyte for 5min before application of ACh (100 μ M) resulted in blockade of the ACh

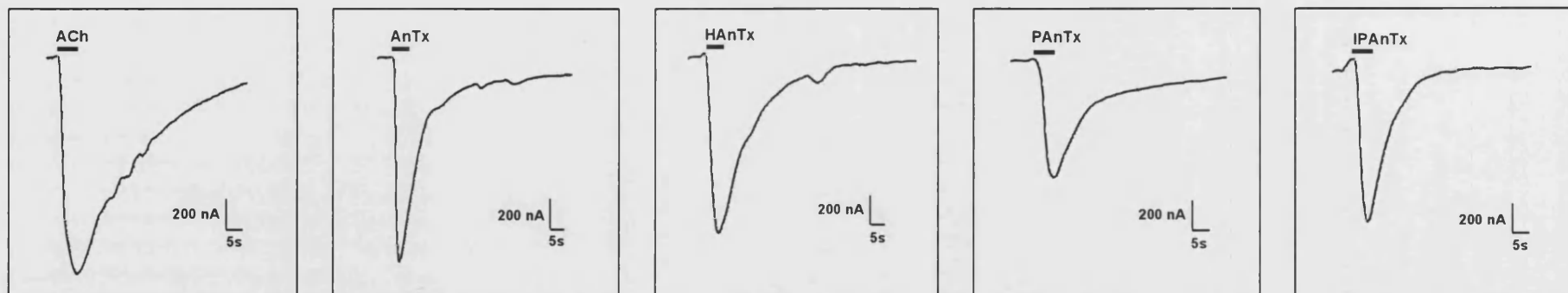
evoked response that was concentration dependent (Figure 5.5). The dose response curve for MLA resulted in an IC_{50} value of $0.24 \pm 0.08 \mu M$.

Current-voltage relationships were determined for ACh ($50 \mu M$) in the absence and presence of $100 nM$ MLA (approximately 50% inhibition). A linear relationship was obtained in the absence of MLA while there was a tendency toward a curvi-linear relationship in the presence of MLA (Figure 5.5). For clarity, no line through the points determining ACh response in the presence of MLA has been drawn.

Recovery from MLA inhibition at a concentration of $100 nM$ was also investigated by challenging with ACh ($100 \mu M$) every minute during MLA washout. Inhibition by MLA was readily reversible, with 85% recovery after washing for 5min (Figure 5.5) and total recovery of responsiveness to ACh within 10min.

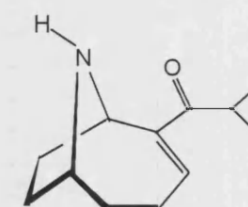
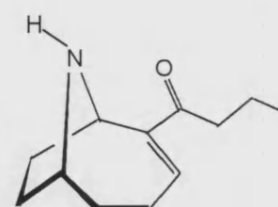
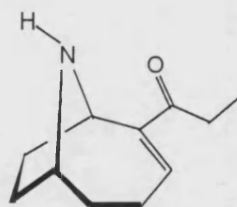
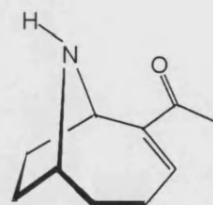
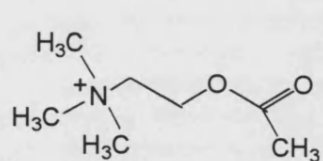


Figure 5.2. *In vitro* transcriptions of mouse muscle nAChR subunits. Radiolabelled transcripts were synthesised as described in section 5.3.1 and analysed on a denaturing gel. Transcripts of α , β , δ and γ subunits are clearly visible.



a)

191



b)

Figure 5.3. Activation of mouse muscle nAChRs expressed in *Xenopus* oocytes by ACh, AnTx and AnTx analogues. a) Representative traces of inward currents elicited by 100 μ M agonist in the same oocyte. b) Chemical structures of ACh, AnTx and AnTx analogues.

a)

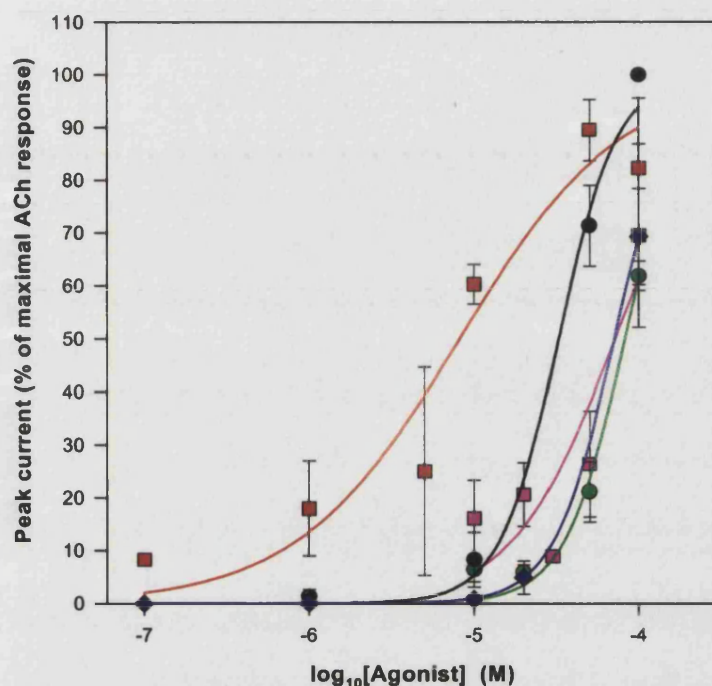
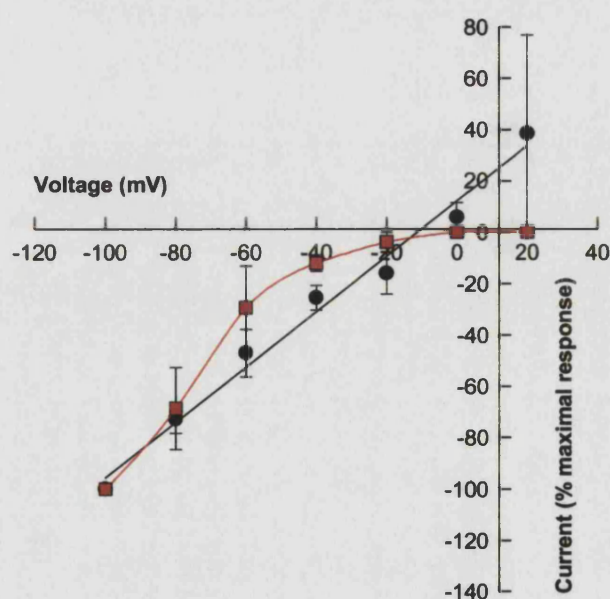


Figure 5.4. Experimental analysis of activation of mouse muscle nAChR expressed in *Xenopus* oocytes by ACh, AnTx and analogues.

a) Dose response curves. Responses to agonist (AnTx (■), HAnTx (◆), PAnTx (●) and IPAnTx (■)) were normalised to the ACh (●) maximum peak height and values from at least three oocytes have been averaged, with sem indicated by vertical bars.

b)



b) Current - voltage relationship for ACh (●) and AnTx (■) at 50 μM concentrations. Holding potential was stepped from -100mV to +20mV. Current responses were normalised with respect to the response observed at -100mV and values from at least three oocytes have been averaged, with sem indicated by vertical bars.

a)

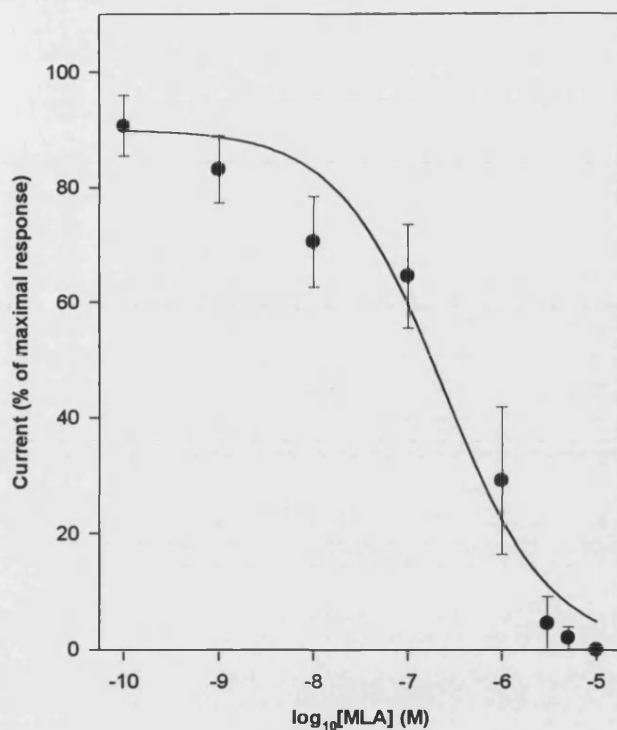
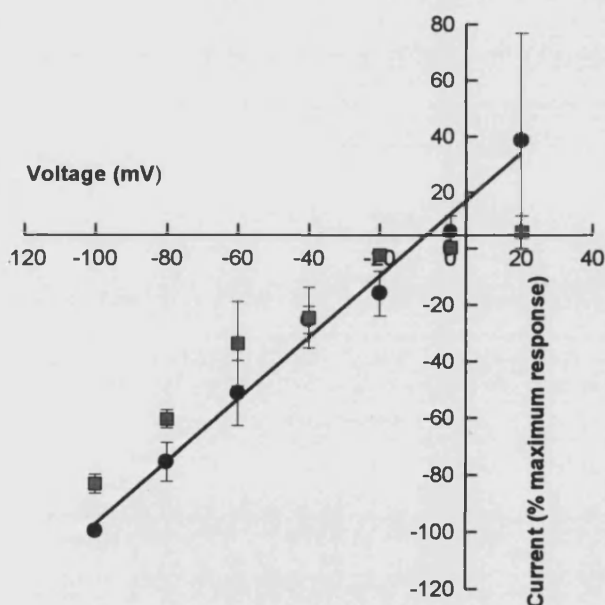


Figure 5.5 Inhibition of mouse muscle nAChRs expressed in *Xenopus* oocytes by MLA. Oocytes were incubated in MLA for 5min and then challenged with a 5s application of ACh with MLA.

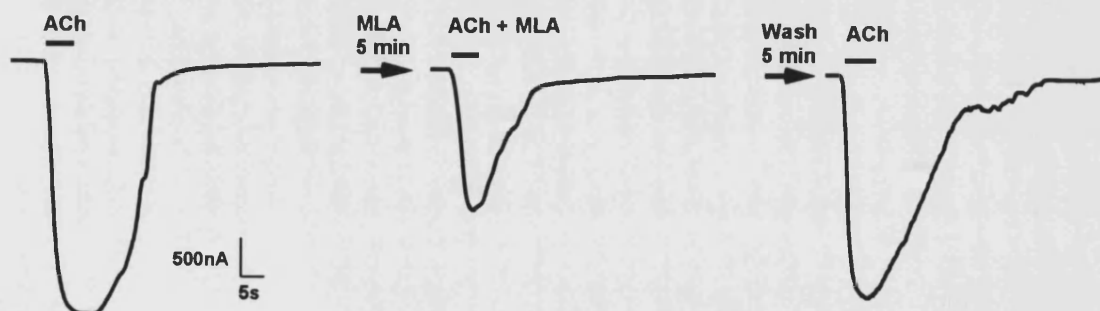
a) Dose response curve for antagonism by MLA of ACh-evoked currents ($100\mu\text{M}$). Responses were normalised to maximum peak height and values from at least three oocytes have been averaged, with sem indicated by vertical bars.

b)



b) Current-voltage relationship for ACh ($50\mu\text{M}$) in the absence (●) and presence (■) of MLA (100nM). Holding potential was stepped from -100mV to $+20\text{mV}$. Current responses were normalised with respect to the response observed at -100mV .

c)



c) Recovery of the ACh response from MLA block. MLA (100nM) was applied to the oocyte for 5min to ensure maximum inhibition. During washout, ACh-evoked currents ($100\mu\text{M}$) were measured periodically.

5.5 Discussion

ACh, AnTx and AnTx analogues were assessed over a range of concentrations on mouse muscle nAChR expressed in *Xenopus* oocytes using two electrode voltage clamp electrophysiology. The EC₅₀ value of 33μM obtained for ACh is in agreement with published figures (Labarca *et al.*, 1995). In comparison, AnTx gave an EC₅₀ value of 8.2μM, which gives a relative potency of AnTx to ACh of 4 to 1. Swanson *et al.* (1986) found that AnTx was 8 times more potent than ACh when measured by muscle contracture in frog skeletal muscle. As Swanson *et al.* used the active enantiomer (+)AnTx, in contrast to the racemic mixture used in the present study, these data are in accord: there is a 150-fold difference in the potency between the (+) and (-) enantiomers (Swanson *et al.*, 1986) and thus, the EC₅₀ values derived in the present study underestimate the potency of the active, (+) enantiomer by 2-fold. Taking this into account, there is excellent agreement between the present and previous study with respect to the relative potency of AnTx, despite the use of frog muscle in Swanson's study and mouse muscle in this study.

In contrast to the muscle nAChR, the α7 subtype of the nAChR is more sensitive to AnTx, giving an EC₅₀ value of 0.58μM (Thomas *et al.*, 1993). On the other hand, ACh was far less potent at the α7 nAChR, giving an EC₅₀ value of 130μM. Thus the relative potency of AnTx to ACh is 225 to 1 on the α7 nAChR. This order of potency agrees with data from whole cell patch clamp of hippocampal neurons exhibiting type 1A currents, which correspond to α7-like nAChR (Alkondon and Albuquerque, 1993; Thomas *et al.*, 1993). Hence, while ACh has a 4-fold greater potency on muscle nAChR than α7 nAChR, AnTx is 14-fold more potent on α7 nAChRs than muscle nAChRs. In other preparations that express different neuronal nAChR (namely, M10 cells that express the chick α4β2 combination and presynaptic nAChR mediating ACh release from hippocampal synaptosomes) AnTx was even more potent (Thomas *et al.*, 1993). From this, it is clear that different nAChR subtypes display a wide range of sensitivities to AnTx but in each case it is more potent than ACh. The potency ratio varies with different subtypes indicative of differences at the agonist binding site.

Studies on AnTx analogues by Swanson *et al.* (1991) and Wonnacott *et al.* (1991) determined that the secondary amine and carbonyl functions of AnTx were crucial for activity. Subsequently, a set of analogues that conserved these features but altered the acetyl side chain by the addition of one or more methine or methylene units was synthesised. Wonnacott *et al.* (1992) demonstrated that the most simple analogue,

HAnTx had one tenth of the activity of AnTx in frog muscle contracture assays. The functional potencies of this set of analogues were assessed on the $\alpha 7$ nAChR (Thomas *et al.*, 1994) and showed less potency than the parent compound. EC₅₀ values for HAnTx and IPAnTx were 0.72 μ M and 0.66 μ M respectively. This gives a rank order of potency for $\alpha 7$ of AnTx > IPAnTx > HAnTx and relative potency values of 1 : 0.88 : 0.80. The data obtained here (see Figure 5.4), on the muscle nAChR gave the following EC₅₀ values: HAnTx 71 μ M, PAnTx 80 μ M and IPAnTx 72 μ M and a rank order of potency of AnTx > HAnTx = IPAnTx > PAnTx. In this case, the analogues are significantly less potent than the parent compound, AnTx, as shown with relative potency values of 1 : 0.12 : 0.10, i.e. AnTx is approximately 10-fold more potent on muscle nAChRs than any of these alkyl modified analogues, agreeing with the frog muscle contracture data (Wonnacott *et al.*, 1992). These data are summarised in Table 5.1.

| Agonist | Mouse muscle (M) | Chick $\alpha 7$ (M) |
|---------|-------------------------------|--|
| ACh | $3.3 \pm 0.43 \times 10^{-5}$ | $1.3 \pm 0.1 \times 10^{-4} \bullet$ |
| AnTx | $8.2 \pm 2.7 \times 10^{-6}$ | $5.8 \pm 0.9 \times 10^{-7} \bullet$ |
| HAnTx | $7.1 \pm 0.69 \times 10^{-5}$ | $7.2 \pm 5.5 \times 10^{-7} \blacklozenge$ |
| PAnTx | $8.2 \pm 0.4 \times 10^{-5}$ | ND |
| IPAnTx | $7.2 \pm 1.7 \times 10^{-5}$ | $6.6 \pm 2.0 \times 10^{-7} \blacklozenge$ |

Table 5.1. EC₅₀ values for mouse muscle and chick $\alpha 7$ nAChRs. Data are mean \pm sem. ND, not determined. \bullet data from Thomas *et al.*, 1993; \blacklozenge data from Thomas *et al.*, 1994.

Molecular modelling studies have shown that the extension of the acetyl side chain does not distort the conformation of the AnTx skeleton (Thomas *et al.*, 1994), thus agreeing with the conservation of biological activity by these analogues at $\alpha 7$ nAChR. However, the comparison detailed above, between the muscle and $\alpha 7$ subtypes shows that while the $\alpha 7$ receptor can evidently accommodate the increased hydrocarbon bulk of the side chain within its binding site, the muscle receptor cannot. Clearly, the binding site of the muscle receptor must differ from the $\alpha 7$ receptor in such a way that the binding of AnTx is preferred over its analogues. Furthermore, it appears that PAnTx, with the most extended side chain has the least affinity for the binding pocket, although there is no significant difference between any of the analogues.

Interestingly, [125 I] α -btX binding assays on rat brain and mouse skeletal muscle have demonstrated that the three analogues bind with higher affinity than AnTx in both tissues (Thomas *et al.*, 1994; Thomas, 1996). In mouse muscle, the rank order of potency according to the measure of [125 I] α -btX binding affinity is IPAnTx > HAnTx > PAnTx > AnTx (Thomas, 1996), while in rat brain it is PAnTx > IPAnTx > HAnTx > AnTx (Thomas *et al.*, 1994). In contrast [3 H]nicotine binding on rat brain gives the rank order of potency as HAnTx > IPAnTx > AnTx > PAnTx (Thomas *et al.*, 1994) suggesting that PAnTx showed a marked preference for the $\alpha 7$ receptor as indicated by [125 I] α -btX binding, being more potent than AnTx and the other analogues and correspondingly less selective for the $\alpha 4\beta 2$ receptor as indicated by [3 H]nicotine binding. This indicated that PAnTx has a preference for the $\alpha 7$ binding site.

Thus, there appear to be discrepancies between potency as measured by binding affinity and as measured by functional assays in *Xenopus* oocytes, suggesting that there is not always a correlation between binding affinity and functional potency. In this study, HAnTx, PAnTx and IPAnTx all bind with high affinity to the α -btX binding site on mouse skeletal muscle but act as weaker agonists at the agonist binding site. It is possible that there may be a difference between organisms as this study used mouse muscle nAChRs whereas Thomas *et al.* (1994) used rat brain nAChRs and/or between developmental stages as mouse muscle binding studies used adult mice (Thomas, 1996) whereas this study used mouse muscle embryonic subunits. Therefore, there would have been replacement of the γ subunit in the embryo with the ϵ subunit in the adult. Moreover, the transcripts encoding the α, β, γ and δ subunits may have undergone different post-translational modifications than those in native mouse muscle. For example, it has been demonstrated that glycosylation of the *Torpedo* nAChR on expression in oocytes is somewhat different to that seen in native *Torpedo* electroplax (Buller and White, 1990b).

The current-voltage relationship obtained for AnTx differed from that for ACh (Figure 5.4). ACh resulted in a linear relationship that showed no signs of inward rectification. However, AnTx showed a distinct deviation from linearity at potentials more positive than -60mV, suggesting that the ion channel was inwardly rectifying. This phenomena is observed as the membrane potential becomes more positive and results in a decrease in current until the channel closes. This tendency for an ion channel to act as a valve, allowing entry of cations and thus inward flux but not exit of cations and thus outward flux is a property more commonly seen with neuronal nAChRs. Indeed, inward rectification is observed for HAnTx and IPAnTx evoked currents on the $\alpha 7$ receptor (Thomas *et al.*, 1994). Muscle nAChRs are not known as inwardly rectifying channels,

suggesting that AnTx was acting as an ion channel blocker in addition to an agonist, as channel blockade is seen as a deviation from linearity in current-voltage relationships (Rozenthal *et al.*, 1989). Interestingly, a deviation from linearity in current-voltage relationships has been observed in neuronally evoked endplate currents and in the spontaneous miniature endplate currents (EPCs) of voltage clamped frog sartorius muscle fibres. However, further analysis has indicated that AnTx does not produce ion channel block on EPCs (Spivak *et al.*, 1980), although in these recordings, the highest concentration of AnTx used was 0.9 μ M, as above this concentration the amplitudes of the evoked currents were too small for reliable measurements. Given that in the present study, an AnTx concentration of 50 μ M was used, the possibility of ion channel block cannot be ruled out.

The antagonist MLA shows remarkable selectivity for the $\alpha 7$ receptor (Wonnacott *et al.*, 1993). Competition binding assays on [125 I] α -btx binding sites on rat brain and frog and mouse muscle showed that MLA had a markedly higher affinity for the rat brain receptor (K_i 1.4 $\times 10^{-9}$ M) than for either the frog muscle receptor (K_i 1.0 $\times 10^{-5}$ M) or the mouse muscle receptor (K_i 3.0 $\times 10^{-7}$ M) (Ward *et al.*, 1990; Thomas, 1996) and thus apparently distinguished between $\alpha 7$ and muscle subtypes, unlike α -btx. Furthermore, it has been shown that the α -btx insensitive receptors, $\alpha 3\beta 2$ and $\alpha 4\beta 2$, when expressed in *Xenopus* oocytes, require micromolar concentrations of MLA for inhibition (Drasdo *et al.*, 1992). IC_{50} values of 0.08 μ M for the $\alpha 3\beta 2$ subtype and 0.65 μ M for the $\alpha 4\beta 2$ subtype were obtained. In comparison, the data obtained in this study gave an IC_{50} value of 0.2 μ M for the muscle nAChR. Thus there appears to be no significant selectivity for MLA between the muscle and the α -btx insensitive neuronal nAChRs. In contrast, recent studies by Palma *et al.* (1996) on the $\alpha 7$ receptor show that this subtype is highly sensitive to inhibition by MLA in the oocyte expression system, giving an IC_{50} value of 25pM. Accordingly, there is a 4000-fold difference in potency of MLA on muscle and $\alpha 7$ receptors. Therefore, functional expression of the muscle and $\alpha 7$ receptor has confirmed earlier observations by Ward *et al.* (1990) that MLA is highly selective for the $\alpha 7$ receptor.

Analysis of the current-voltage relationships of ACh in the absence and presence of MLA (Figure 5.5) suggested that there was not a deviation from linearity over the range -100 to -20mV. However, at more positive potentials, there appeared to be a slight deviation from linearity. But in view of the overlapping error bar at +20mV (in comparison with that for AnTx, Figure 5.4) there is no evidence to suggest that inward rectification is occurring or that MLA acts as a channel blocker. Furthermore, Alkondon *et al.* (1992) have

demonstrated that the antagonism of ACh and AnTx-evoked currents by MLA in hippocampal neurons is voltage independent, supporting a competitive mode of antagonism. This was also reinforced by the finding that single channel open times of type 1A channels were not affected by MLA, indicating that MLA inhibited the nAChR in the closed conformation. That MLA is a competitive antagonist is also supported by ligand binding studies (Wonnacott *et al.*, 1993), including those presented in Chapter 3 on *Manduca*, where MLA was a potent inhibitor of α -btx binding.

In summary, this study shows that the muscle nAChR is activated by AnTx but to a lesser extent by its analogues, in contrast with the $\alpha 7$ nAChR. Furthermore, MLA has a weaker effect on the muscle nAChR than the $\alpha 7$ nAChR. Thus these compounds demonstrate the utility of neurotoxins as pharmacological tools for characterising nAChR subtypes and would therefore have been ideal ligands for characterising the α -like subunit of the nAChR from *Manduca*, MARA1. The expression of MARA1 in *Xenopus* oocytes (assuming that it was able to form a functional homomeric receptor) would have conclusively identified MARA1 as a modified (or not) subunit of the nAChR, on application of nicotine and comparison with ACh. AnTx and its analogues would have extended the pharmacological profile, while blockade of agonist-evoked currents by α -btx and MLA would have confirmed its nAChR status and allowed comparison with the vertebrate $\alpha 7$ subunit.

APPENDIX 1

Torpedo californica α subunit sequence

-24 MILCSYWHVGLVLLLFSCCGLVLGSEHETRLVANLLENYNKVIRPVEHHT
27 HFVDITVGLQLIQLISVDEVNQIVETNVRLRQQWIDVRLRWNPADYGGIK
77 KIRLP SDDV**W**LPDLVL**Y**NNADGDFAIVHMTKLLLDYTGKIMWTPPAIFKS
127 YCEIIVTHFPFDQONCTMKLGI**W****T**YDGTKVSI SPESDRPDLSTFMESGEW
177 VMKDYRGWKHWV**Y****T**CCPDTP**Y**LDITYHFIMQRIPLYFVVNVIIPCLLES
TM1
227 FLTGLVFYLPTDSGEKMTLSISVLLSLTVFLLVIVELIPSTSSAVPLIGK
TM2
277 YMLFTMIFVISSIIITVVVINTHHRSPSTHTMPQWVRKIFIDTIPNVMFF
TM3
327 STMKRASKEKQENKIFADDIDISDISGKQVTGEVIFQTPLIKNPDVKSAI
377 EGVKYIAEHMKSDEESSNAAEEWKYVAMVIDHILLCVFMLICIIGTVSVF
TM4
427 AGRLIELSQEG

Underlined letters correspond to putative transmembrane domains; italic letters correspond to the cys loop; bold letters correspond to residues postulated to be involved in ligand binding.

From Noda *et al.* (1982, 1983b). SwissProt accession number: p02710.

APPENDIX 2

Primers

a) Location of primers on fragment c (from Clarke, 1991).

Primer sequences in bold. Sense primers underlined, antisense primers italicised. Sequences that are both underlined and in italics represent overlapping sense and antisense primers. Location of degenerate primers used to find other α -like subunits indicated as dotted lines beneath amino acid sequence.

| | | | | | |
|------|---|--|--|--|-----|
| 1 | CGGCCGCGCGAGTACGGCGGCGGTGGAAATGCTGCACGTGCCTTCCGACCACATC | <u>TGGCGGCCGACATCGTGCTT</u> | 75 | | |
| 1 | R P R E Y G G V E M L H V P S D H I W R P D I V L | | 25 | | |
| 76 | <u>TACAACAATGCTGACGGCAACTTT</u> | GAGGTGACGCTGGCGACTAAGGCGACGCTCAATTACACCGGCCGAGTGGAG | 150 | | |
| 26 | Y N N A D G N F E V T L A T K A T L N Y T G R V E | | 50 | | |
| 151 | TGGCGTCCGCCCCCATCTACAAGTCTCGTGCGAGATCGACGTAGAGTACTTTCCATTTCGACCAGCAGACGTGC | | 225 | | |
| 51 | W R P P A I Y K S S C E I D V E Y F P F D Q Q T C | | 75 | | |
| 226 | GTCATG <u>AAGTTTGGCTCGTGGACCTAC</u> | GACGGGTTTCAGGTAGATCTGCGACATATCGATGAA | <u>GTGCGAGGCACC</u> | 300 | |
| 76 | V M K F G S W T Y D G F Q V D L R H I D E V R G T | | 100 | | |
| 301 | <u>AACGTCGTG</u> | GAGT | <u>TTGGGCGTCGACCTCTCCGAGTTT</u> | TACACCTCTGTGAGTGGGACATCCTGGAGGTGCCCGCC | 375 |
| 101 | N V V E L G V D L S E F _ Y _ _ T _ S _ V _ E _ W _ D _ I _ L E V P A | | 125 | | |
| 376 | GTCCGAAATGAA <u>AAGTTCTACACGTGCTGCGAC</u> | GAGCCCTATCTAGATATAACTTTCAACATCACGATGCGCCGC | | 450 | |
| 126 | V R N E K F Y T C C D E P Y L D I T F N I T M R R | | 150 | | |
| 451 | AAAACACTCTTCTACACCGTTAATCTGATCATTCGTCATGGGAATATCGTTTCTGACGGTCTTGGTATTTTAC | | 525 | | |
| 151 | K T L F Y T V N L I I P C M G I S F L T V L V F Y | | 175 | | |
| 526 | TTGCCTTCGGACAGTGGC | <u>GAGAAGGTATCGCTGTCC</u> | ATATCGATCCTACTGTCGCTCACCGTGTCTTCTCTGCTG | 600 | |
| 176 | L P S D S G E K V S L S I S I L L S L T V F F L L | | 200 | | |
| 601 | TTGGCCGAGATCATTCACCGACTTCGCTGGTGGTGCCACTGCTTGGCAAGTTTGTCTCTTACCATGATC | <u>CTC</u> | | 675 | |
| 201 | L A E I I P P T S L V V P _ L _ _ L _ G _ K _ F _ V _ L _ F _ T M I L | | 225 | | |
| 676 | <u>GATACGTTACGATATGCGTG</u> | ACTGTGGTGGTATTGAATGTGCACTTCCGCTCGCCACAGACGCACACAATGTGC | | 750 | |
| 226 | D T F S I C V T V V V L N V H F R S P Q T H T M S | | 250 | | |
| 751 | CCGTGGGTGCGGCGCGTGTTCATCCACGTGCTGCCGCGGCTCCTGGTCATGCGCGGCCCGCACTACCGACTCGAC | | 825 | | |
| 251 | P W V R R V F I H V L P R L L V M R R P H Y R L D | | 275 | | |
| 826 | CCGCACCGC | <u>AGTCGCTTCGCAGGACTGGTGACC</u> | GGCGCTGGCGAGACCACACTATGGGACGAGGGCTCGCCGGGC | 900 | |
| 276 | P H R S R F A G L V T G A G E T T L W D E G S P G | | 300 | | |
| 901 | GTGCCGGCTCCGCCCGTCCGCCGCCCTGCGCACCCCGCTGGCGCCCTGCGCCGCTGCGCGCCCGCTGAGGCG | | 975 | | |
| 301 | V P A P P R P P P C A P P L A P C A A C A P A E A | | 325 | | |
| 976 | CCGGCGCTCTGCGACGCGTGC | CGCGGTGGCACCGGTGTCCCAGCTACATAAGGCTATTGACGGCATCAATTAT | | 1050 | |
| 326 | P A L C D A L R R W H R C P E L H K A I D G I N Y | | 350 | | |
| 1051 | ATTGCCGACCAAACTAGGAAAGAGGAAGAGTCCACTAGGGTGAAAGAGGACTGGAAGTACGTAGCG | <u>ATGGTACTC</u> | | 1125 | |
| 351 | I A D Q T R K E E E S T R V K E D W K Y V A M V L | | 375 | | |
| 1126 | <u>GACAGACCATTCTCTG</u> | TGGATATTCACGCTAGCGGTGGTGGTGGGCTCGGCGGGCATCATCCTGCAGGCGCCGACG | | 1300 | |
| 376 | D R P F L W I F T L A V V V G S A G I I L Q A P T | | 400 | | |
| 1301 | CTGTACGACGAGCGCGCGCCCATCGACGTGCGC | <u>CTGTCCGAGATAGCGTACACGGCC</u> | GCCAAGCCGCGCCACCG | 1375 | |
| 401 | L Y D E R A P I D V R L S E I A Y T A A K P R P P | | 425 | | |
| 1376 | CCGCCACGCTGAGCGGGTCTCGCGTCACGTGCTACGTGTACGCGTGACGCGTCACGCATCAGCGTCA | | 1450 | | |
| 426 | P P R | | 428 | | |
| 1451 | GGCGTCTCTCGACGGGCTCCAGTGATTCTCGCTCGTATTTTCGATTACGCAAATTCGAATTTGCAATATTGTAC | | 1525 | | |
| 1526 | ATAGTATACATAATTATTATAGTTTATACAAAGAAACACCGCGATCGATTCTAAGTCGAATTTCTTTTGATAGAG | | 1600 | | |
| 1601 | <u>ACGAAATAAAATGATCGTTGTAT</u> | | 1624 | | |

b) Location of primers on fragment a.

Primer sequences in bold. Sense primers underlined, antisense primers italicised. Sequences that are both underlined and in italics represent overlapping sense and antisense primers.

```

1  GTGCGAGGCCCAACGTGTGGAGTTGGGCGTCAACTCATGCGGAGTGTGACAAAAATATTAC 60
-21                               M R S V T K Y Y -14

61  TTACATGGTGTGTTTGTGTTTGGACAGGATGTGCCGGCAACCCGACGCGAAGCGGCTG 120
-13  L H G V V L F A T G C A G N P D A K R L 7

121 TACGACGATCTGCTCAGCAATTACAACAAGTTGGTGCGGCCTGTACTCAACGTCAGCGAC 180
8  Y D D L L S N Y N K L V R P V L N V S D 27

181 GCGCTCACCGTGCGCATCAAGCTCAAGCTGAGCCAGCTCATCGACGTGTCAGCCGCCTT 240
28  A L T V R I K L K L S Q L I D V 43

241 TAACACAAAACGTGACAATCACTAGTTCTTACAATATCATACTGGAATCGTTGCAATAA 300

301 TAATAGCAATAGTTTAAACATTGTAATATAATTGTTGCAGAATCTCAAGAATCAAATAATG 360
44                               N L K N Q I M 50

361 ACAACCAATCTATGGGTGGAACAGGTTAGTCTCATTGTGTATGTGTTGCACGATTATTG 420
51  T T N L W V E Q 58

421 CAAGTTGCATACAGTTTTTCTAGTAGCTATTGTGTTATTCGAGAGCTGGTACGACTAC 480
59                               S W Y D Y 63

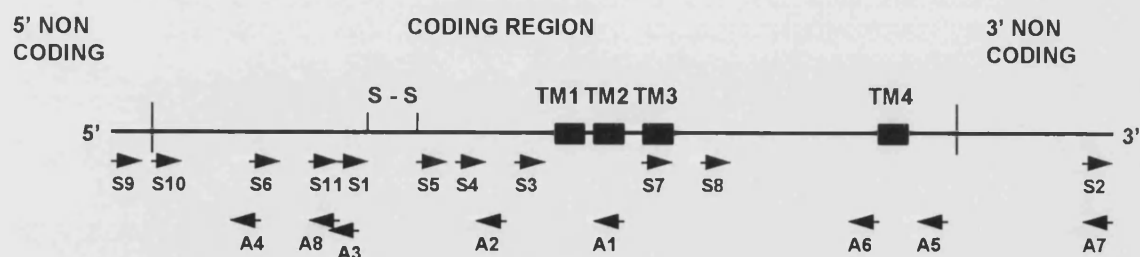
481 AAGCTTTCGTGGGAGCCGCGAGTACGGCGGCGTGGAAATGCTGCACGTGCCTTCCGAC 540
64  K L S W E P R E Y G G V E M L H V P S D 83

541 CACATCTGGCGGCCCGACATCGTGCTTTACAACAATGCTGAC 582
84  H I W R P D I V L Y N N A D 97

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c) Schematic of MARA1 showing approximate location of primers.

S, sense primer; A, antisense primer. Numbering consistent with text in Chapter 2. TM, transmembrane domain; S-S, cys loop. Not to scale.



d) Calculation of annealing temperature of primer.

Annealing temperature (T_m) of primer calculated using the equation: $T_m = (A+T)2 + (G+C)4$.

APPENDIX 3

Solutions

2YT Medium

1.6% (w/v) bacto-tryptone
1.0% (w/v) bacto-yeast extract
0.5% (w/v) NaCl
pH 7.0

NZY medium

1.0% (w/v) NZ amine
0.5% (w/v) NaCl
0.5% (w/v) bacto-yeast extract
0.2% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
pH 7.0

For agar plates, 1.5% (w/v) bacto-agar added. For top agarose, 0.7% (w/v) agarose added.

SM

0.58% (w/v) NaCl
0.2% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
5% (v/v) 1M Tris-Cl pH7.5
0.5% (v/v) 2% gelatin solution

SSC (20x stock solution)

3M NaCl
0.3M sodium citrate
pH7.0

Denhardt's solution (50x stock solution)

1% (w/v) Ficoll (type 400)
1% (w/v) polyvinylpyrrolidone
1% (w/v) BSA

TE

10mM Tris-Cl pH 7.8
1mM EDTA pH 8.0

TAE

40mM Tris-acetate
1mM EDTA pH8.0

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